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THE EQUILIBRIUM BETWEEN HEMOGLOBIN AND OXYGEN IN WHOLE AND HEMOLYZED BLOOD OF THE TAUTOG, WITH A THEORY OF THE HALDANE EFFECT

R. W. ROOT AND LAURENCE IRVING[1]

(From the College of the City of New York; Swarthmore College; and the Marine Biological Laboratory, Woods Hole)

The combination of oxygen with the blood of certain fishes occurs far less readily when the CO_2 tension is raised, but in a number of these same fishes hemolysis largely eliminates the sensitivity of the hemoglobin toward CO_2 (Black and Irving, 1938; Root, Irving, and Black, 1939). The peculiar effect of hemolysis indicates that the properties of the whole blood, as regards O_2-combination in the presence of CO_2, do not parallel the properties of the hemoglobin when released from the cell. One cannot, therefore, infer the properties of fish hemoglobin in their entirety from a study of whole blood alone. It has been further shown that the whole and hemolyzed blood of the tautog not only differed with respect to O_2-combination in the presence of CO_2, but the reciprocal effect of oxygenation on CO_2-combination showed significant differences (Root and Irving, 1940). In hemolyzed blood the ratio $\frac{-\Delta BHCO_3}{\Delta O_2}$ is apparently constant for any given CO_2 tension, whereas this is not the case in whole blood. The behavior of the whole blood is apparently exceptional, since it is commonly considered that for any given hemoglobin $\frac{-\Delta BHCO_3}{\Delta O_2}$ is constant (Henderson, 1928; Redfield, 1933*a*).

The material to be presented in this paper is in part an amplification of the work done by us on the blood of the tautog, *Tautoga onitis* (Linn.). The equilibrium between hemoglobin and oxygen in both whole and hemolyzed blood has been examined in detail over a wide range of CO_2 tensions. From the study a clearer picture has developed

[1] The authors are indebted to the U. S. Bureau of Fisheries at Woods Hole for the provision of laboratory space and facilities during the course of this investigation.

of the contrast in behavior of whole and hemolyzed fish blood. It has enabled us to describe theoretically not only the equilibrium that exists between hemoglobin and oxygen in the two conditions of the blood, but also to give an interpretation of the effect of oxygenation on CO_2-combination (Haldane effect) as observed in the blood of this fish.

Throughout this investigation the methods of handling the blood, equilibrating it, and analyzing the gas phases were the same as those described in the paper by Root and Irving (1940).

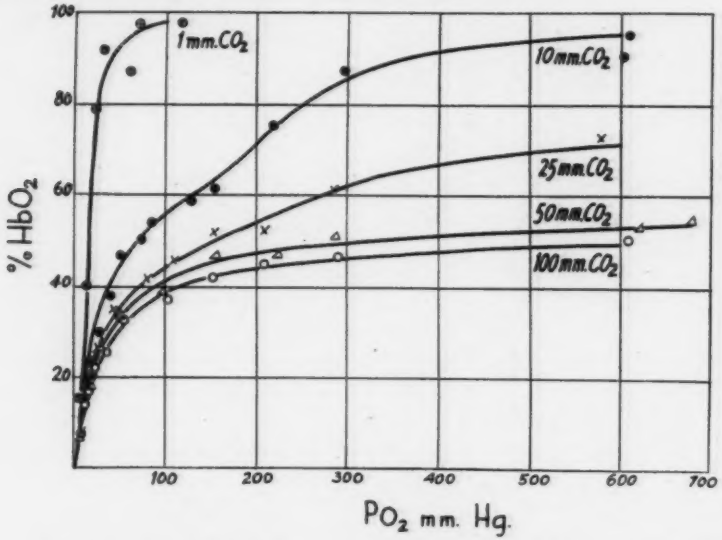

FIG. 1. Oxygen dissociation curves of whole tautog blood at 15° C. and constant CO_2-tensions. The curves have been drawn according to the equation indicated in the text, using the following constants:

PCO_2 mm. Hg.	α_1	α_2	α_4	α_8	K_1	$K_3 \times 10^3$	$K_4 \times 10^9$
0–1	0	1.0	0	0		.51	
10	.75	0	.25	0	.0286		.25
25	.60	0	.15	.25	.0286		.16
50	.56	0	0	.44	.0286		
100	.52	0	0	.48	.0286		

COMPARISON OF THE O_2-DISSOCIATION CURVES OF WHOLE AND HEMOLYZED BLOOD

The O_2-dissociation curves for both whole and hemolyzed blood of the tautog have been established at 10, 25, 50, and 100 mm. CO_2

tension. The former are shown in Fig. 1 along with a curve established in the virtual absence of CO_2 (data of Root, Irving and Black, 1939). Those for hemolyzed blood are presented in Fig. 2. Each curve represents the data from a single large sample of blood, with the exception of the one at 100 mm. CO_2 where two lots of blood were used. The curves at each CO_2 tension have been drawn from an equation which seemed best to fit the data, the constants used being shown in the table

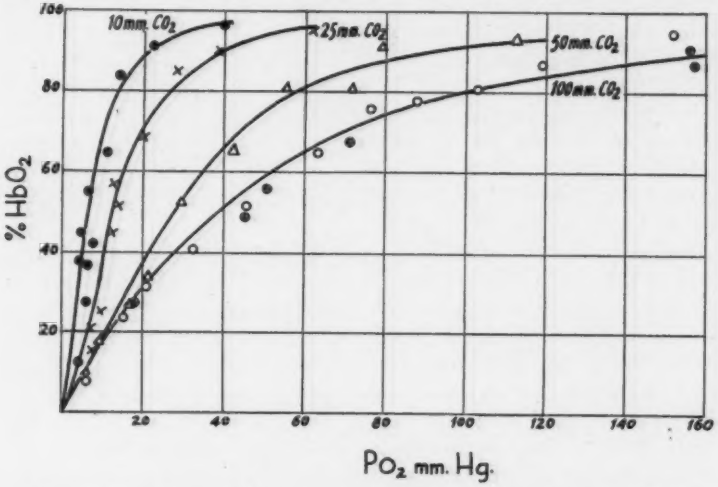

FIG. 2. Oxygen dissociation curves of hemolyzed tautog blood at 15° C. and constant CO_2-tensions. The curves have been drawn according to the equation given in the text, using the following constants:

PCO_2 mm. Hg.	α_1	α_2	α_3	K_1	$K_2 \times 10^2$
10	0	1.0	0		2.0
25	0	1.0	0		.59
50	.2	.8	0	.10	.11
100	.5	.5	0	.05	.033

beneath the figures. Reference to this equation and its implications will be made later.

If one compares in a general way the family of curves obtained for each type of blood the following major differences become evident: (1) the hemolyzed blood has a greater affinity for oxygen at comparable CO_2 tensions than has the whole blood; (2) the dissociation curves for hemolyzed blood at comparable CO_2 tensions are different in shape from those of whole blood; (3) the hemolyzed blood, up to 100 mm. CO_2

tension, shows no evidence of hemoglobin inactivation, whereas whole blood does; and (4) the reduction in affinity for oxygen with rise in CO_2 tension is different in magnitude in the two types of blood.

In order to further the comparison of the behavior of the two types of blood it is desirable to study as closely as possible the shape of the O_2-dissociation curves in each case. For our purpose this has consisted in an attempt to fit certain existing equations describing the equilibrium between hemoglobin and oxygen to the data for the curves. Once a suitable fit is obtained the theoretical implications of the particular equation employed offer some insight into the behavior of the hemoglobin at any particular CO_2 tension. Any attempt to fit existing equations to dissociation curves established at constant CO_2 tension, instead of constant pH, is perhaps open to criticism, but we have reason to believe that the results are not so totally different that the general conclusions drawn from such an analysis would be invalidated when the pH is kept constant.

It is obvious from examination of the data that the classical equation of Hill (1910) is too simple an expression adequately to describe all of the dissociation curves. We have therefore resorted to the equation suggested by Redfield (1933*b*) and used by Green and Root (1933) in describing the equilibrium between hemoglobin and oxygen in certain fish bloods. This equation is based on the theory that there are different components of the respiratory protein which act independently of each other in compliance with Hill's equation but with different values of n. If the O_2-dissociation constants of the components having values of n of 1.0, 2.0, 3.0, 4.0 etc., are designated by K_1, K_2, K_3, K_4, etc. and the fraction of the total O_2 bound by each of these components as α_1, α_2, α_3, α_4, etc., the fraction of the total respiratory protein present in the oxygenated form, Y, at any particular O_2-tension, χ. is given by the equation:

$$Y = \frac{\alpha_1 K_1 \chi^1}{1 + K_1 \chi^1} + \frac{\alpha_2 K_2 \chi^2}{1 + K_2 \chi^2} + \frac{\alpha_3 K_3 \chi^3}{1 + K_3 \chi^3} + \frac{\alpha_4 K_4 \chi^4}{1 + K_4 \chi^4}.$$

It is necessary in tautog whole blood to introduce the term α_0 to take into account the fraction of hemoglobin inactivated at high CO_2 tensions. The sum of α_0 plus the other fractions will equal 1. Fortunately it has not been necessary for us to use more than two terms of the general equation in describing the more complicated dissociation curves obtained with the blood of this fish. The simple curves require but one term and could as well be described with Hill's equation, providing we take into account the fraction of hemoglobin inactivated in whole blood at high CO_2 tensions.

The general results of our analysis of the O_2-dissociation curves, using the above equation, can be obtained by reference to the table of constants beneath Figs. 1 and 2, and to the curves drawn according to the equation, using these constants. It is clear that in whole tautog blood the O_2-dissociation curve in the virtual absences of CO_2 is sigmoid and is characterized by a value of $n = 2$, i.e. there is a single component of the hemoglobin uniting with two molecules of oxygen at a time. As CO_2 is added the dissociation curve not only moves to the right but becomes more complicated. Components with different values of n and dissociation constants sufficiently different to produce definite undulations in the curve come into view. Further addition of CO_2 brings about inactivation of some of the hemoglobin and finally simplifies the O_2-dissociation curve to the form of a rectangular hyperbola, i.e. there is now a single component of the hemoglobin combining with one molecule of oxygen at a time. We see, then, that CO_2, in addition to inactivating a portion of the hemoglobin, completely changes the O_2-dissociation curve of whole blood from a second to a first power curve, and that the intermediate stages in the conversion apparently produce different components that unite with different amounts of oxygen at a time, thus complicating the dissociation curves in this region.

The picture presented is essentially that obtained earlier by Green and Root (1933) on the same blood at 25° C. It differs in that their intermediate curves did not show the marked inflections that ours show. However, a too rigorous comparison of the intermediate curves is not justified since ours were established at a constant CO_2 tension instead of constant pH, as theirs were, and at 15° C. instead of 25° C.

In hemolyzed blood, as the table beneath Fig. 2 will indicate, not only are the dissociation constants for the curves much larger than those for whole blood at comparable CO_2 tensions, but those components characterized by a value of n greater than 1 persist at CO_2 pressures at which they have definitely disappeared in whole blood. Furthermore, it can be seen that there is no necessity for assuming that any of the hemoglobin has become inactive, as was the case in whole blood. There is this similarity, however, between whole and hemolyzed blood: added CO_2 decreases the magnitude of the dissociation constants (Bohr effect) and changes the behavior of the hemoglobin in the direction of components which react with only a single molecule of oxygen at a time ($n = 1$). The latter process requires a much higher CO_2 tension in the hemolyzed blood, not being completed even at 100 mm. CO_2 tension.

By way of summary, the study of the O_2-dissociation curves of

whole and hemolyzed blood, both comparatively and individually, has yielded sufficient information to enable us to visualize, at least partially, what the addition of CO_2 does to the hemoglobin of this fish. In whole blood it changes the dissociation constants, modifies the behavior of the components combining with oxygen, and effects considerable inactivation of the hemoglobin. In hemolyzed blood, at least up to 100 mm. CO_2, we obtain the first two effects, but not the latter. However, the dissociation constants are all much larger in magnitude, and the change in the components of the hemoglobin requires a much higher tension of CO_2. We are confronted then with the apparent fact that liberation of the hemoglobin from the cell, in the presence of CO_2, in some way decreases the dissociation of oxygen from the hemoglobin, prolongs the existence of those components of the hemoglobin which act as if they were combining with more than one molecule of oxygen at a time, and abolishes, or greatly postpones, any inactivation of the O_2-combining groups. The reason for this is yet to be elucidated.

Theoretical Interpretation of the Effect of Oxygenation on the CO_2 Bound by the Blood (Haldane Effect)

In a previous paper (Root and Irving, 1940) evidence was presented to show that the effect of oxygenation on CO_2 transport in tautog blood was different from that in the blood of mammals. It was tentatively suggested that a part of the difference might be explained on the basis of the theory that there were several O_2-combining components of the hemoglobin behaving differently with respect to O_2-combination and CO_2 sensitivity. At the time we could not see how such an interpretation could apply to hemolyzed blood, since the Haldane effect here was quite typical, and suggested that perhaps there were fundamental changes in the properties of hemoglobin upon hemolysis. With the combined picture we now have of the effect of CO_2 on the O_2-combining power and the reciprocal effect of oxygenation on the CO_2-combining power, we are in a position to give a more adequate interpretation of the Haldane effect as observed in this blood.

The primary fact to be explained is the inconstant $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio found at 10 and 25 mm. CO_2 pressure for whole blood. To those familiar with mammalian blood it is well known that these ratios are considered to be constant for any single hemoglobin, and it is usually believed that they are constant, though of different magnitude, for the hemoglobin of any species (Redfield, 1933*a*). An inconstant ratio, then, would be considered atypical as compared with the usual constant ratios.

Our interpretation rests on the fundamental postulate that the hemoglobin consists of O_2-combining components which can combine either with a single molecule of oxygen at a time, or more, depending upon how many prosthetic groups the components contain. On this basis let the following assumptions be made:

1. For any single O_2-combining component the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio is constant. Let this constant be called R.

2. Different O_2-combining components have different values for R. This is not a groundless assumption for R varies among hemoglobins of different species (Redfield, 1933*a*).

3. Hence, if the proportions of the several O_2-combining components change, the ratio $\frac{-\Delta BHCO_3}{\Delta O_2}$ for the combined components of the whole hemoglobin *may be* inconstant.

We are now in a position to apply these assumptions. Let the components of the hemoglobin be designated as α_1, α_2, α_3, α_4 etc. in accordance with the previous treatment (see page 310), and the corresponding $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratios be written as follows:

$$\frac{-\Delta BHCO_3^{\alpha_1}}{\Delta O_2^{\alpha_1}} = R_{\alpha_1}; \qquad \frac{-\Delta BHCO_3^{\alpha_2}}{\Delta O_2^{\alpha_2}} = R_{\alpha_2};$$

$$\frac{-\Delta BHCO_3^{\alpha_3}}{\Delta O_2^{\alpha_3}} = R_{\alpha_3}; \qquad \frac{-\Delta BHCO_3^{\alpha_4}}{\Delta O_2^{\alpha_4}} = R_{\alpha_4}. \quad (1)$$

Any given increment of oxygenation of the whole hemoglobin, ΔO_2^{α}, will be equal to the sum of the increments for each of the components, i.e.

$$\Delta O_2^{\alpha} = \Delta O_2^{\alpha_1} + \Delta O_2^{\alpha_2} + \Delta O_2^{\alpha_3} + \Delta O_2^{\alpha_4}. \quad (2)$$

The base correspondingly released by the whole hemoglobin, $-\Delta BHCO_3^{\alpha}$, will be equal to the sum of that released by each of the components, i.e.

$$-\Delta BHCO_3^{\alpha} = -\Delta BHCO_3^{\alpha_1} + -\Delta BHCO_3^{\alpha_2} + -\Delta BHCO_3^{\alpha_3} + -\Delta BHCO_3^{\alpha_4}. \quad (3)$$

Combining equations (2) and (3) we have

$$\frac{-\Delta BHCO_3^{\alpha}}{\Delta O_2^{\alpha}} = \frac{-\Delta BHCO_3^{\alpha_1} + -\Delta HBCO_3^{\alpha_2} + -\Delta BHCO_3^{\alpha_3} + -\Delta BHCO_3^{\alpha_4}}{\Delta O_2^{\alpha_1} + \Delta O_2^{\alpha_2} + \Delta O_2^{\alpha_3} + \Delta O_2^{\alpha_4}}. \quad (4)$$

It is evident from (1) that equation (4) can be rewritten in the following form:

$$\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}} = \frac{R_{\alpha_1}\cdot\Delta O_2^{\alpha_1} + R_{\alpha_2}\cdot\Delta O_2^{\alpha_2} + R_{\alpha_3}\cdot\Delta O_2^{\alpha_3} + R_{\alpha_4}\cdot\Delta O_2^{\alpha_4}}{\Delta O_2^{\alpha_1} + \Delta O_2^{\alpha_2} + \Delta O_2^{\alpha_3} + \Delta O_2^{\alpha_4}}. \quad (5)$$

By the use of this fundamental equation curves can be drawn which relate the total CO_2 to the degree of oxygenation of the hemoglobin. Since the effect of oxygenation upon CO_2-combination is called the Haldane effect, the curves which describe the effect of change in combined oxygen (ΔO_2) upon the combined CO_2($\Delta BHCO_3$) will be called Haldane curves. These curves have been constructed from data calcu-

TABLE I

Data for construction of Haldane curve for tautog whole blood at 10 mm. CO_2. $R_{\alpha_1} = .05$; $R_{\alpha_4} = .135$; $\Delta O_2^{\omega} = 10$ per cent HbO_2.

HbO_2	$\Delta O_2^{\alpha_1}$	$\Delta O_2^{\alpha_4}$	$-\Delta BHCO_3^{\alpha_1}$	$-\Delta BHCO_3^{\alpha_4}$	$-\Delta BHCO_3^{\omega}$	Total $[CO_2]$
per cent	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
0	0	0	0	0	0	20.50
10	10	0	.50	0	.50	20.00
20	10	0	.50	0	.50	19.50
30	10	0	.50	0	.50	19.00
40	10	0	.50	0	.50	18.50
50	10	0	.50	0	.50	18.00
60	8.5	1.5	.43	.20	.63	17.37
70	5.0	5.0	.25	.68	.93	16.44
80	2.5	7.5	.13	1.01	1.14	15.30
90	2.0	8.0	.10	1.08	1.18	14.12

lated according to the principle of equation (5) and presented in Tables I and II. The change in oxygenation is expressed as an increment of the percentage saturation of the whole hemoglobin, since the original O_2-dissociation curves are drawn in that manner; furthermore we have given ΔO_2^{ω} the arbitrary value of 10% HbO_2. This is a sufficiently small increment to provide an adequate number of points on a theoretical Haldane curve. It is to be understood that in assigning a value of 10% HbO_2 to ΔO_2^{ω} it means that the fully reduced hemoglobin is oxygenated in steps of 10 per cent and for each step the $BHCO_3^{\omega}$ released is calculated according to equation (5). The value obtained when subtracted from the total CO_2 remaining in the preceding step of oxygenation will provide a point on the Haldane curve.

By breaking down the O_2-dissociation curve at any given CO_2 pressure into its components (see Figs. 3 and 5) the values for the

ΔO_2 of the components can readily be determined for any value of $\Delta O_2{}^{\omega}$. In addition to knowing these values, the R values for each of the components must be known in order to calculate the $BHCO_3{}^{\omega}$ released on oxygenation of the hemoglobin. These can be determined from the slope of the experimentally established Haldane curve providing there is any part of it where the slope is due to only one component acting. The latter can be determined by consulting the corresponding O_2-dissociation curves for the components. Fortunately we have had to deal with only two components at any one time and this has simplified the work of calculating the R values. Once the value for one component is known, the other can be readily determined.

As an example of the determination of the R values, we refer to the whole blood of the tautog at 10 mm. CO_2 pressure where there are two

TABLE II

Data for construction of Haldane curve for tautog hemolyzed blood at 100 mm. CO_2.
R_{α_1} = .04; R_{α_2} = .06; $\Delta O_2{}^{\omega}$ = 10 per cent HbO_2.

HbO_2	$\Delta O_2{}^{\alpha_1}$	$\Delta O_2{}^{\alpha_2}$	$-\Delta BHCO_3{}^{\alpha_1}$	$-\Delta BHCO_3{}^{\alpha_2}$	$-\Delta BHCO_3{}^{\omega}$	Total $[CO_2]$
per cent	*per cent*	*per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*
0	0	0	0	0	0	35.80
10	10	0	.40	0	.40	35.40
20	8.0	2.0	.32	.12	.44	34.96
30	5.0	5.0	.20	.30	.50	34.46
40	4.0	6.0	.16	.36	.52	33.94
50	4.0	6.0	.16	.36	.52	33.42
60	3.5	6.5	.14	.39	.53	32.89
70	3.0	7.0	.12	.42	.54	32.35
80	3.0	7.0	.12	.42	.54	31.81
90	3.0	7.0	.12	.42	.54	31.27

components, α_1 and α_4 acting. By examining the O_2-dissociation curves for these components, as shown in Fig. 3, it becomes evident that below 50 per cent O_2-saturation of the whole hemoglobin the α_4 component is contributing nothing to the O_2-saturation of the hemoglobin. Therefore the slope of the corresponding Haldane curve in this region is due solely to the α_1 component, i.e.:

$$\frac{-\Delta BHCO_3{}^{\omega}}{\Delta O_2{}^{\omega}} = \frac{-\Delta BHCO_3{}^{\alpha_1}}{\Delta O_2{}^{\alpha_1}} = R_{\alpha_1}.$$

The value for R_{α_1} when $\Delta O_2{}^{\alpha_1}$ is put on a percentage O_2-saturation basis, turns out to be equal to 0.05 at this particular CO_2 tension. To calculate R_{α_4} one may go to a position of the Haldane curve where both components are clearly contributing to the slope of the curve. Between 60 per cent and 90 per cent O_2-saturation there is such a region.

Again, by consulting the O_2-dissociation curves for the components, one can find just how much of this 30 per cent increment of O_2-saturation is due to each of them. It happens that approximately one-third (10 per cent HbO_2) is contributed by the α_1 component, and the rest (20 per cent HbO_2) by the α_4 component. R_{α_4} may now be found as follows:

$$-\Delta BHCO_2^{\omega} = 3.2 \text{ vol. per cent (from experimental Haldane curve)}$$
$$-\Delta BHCO_2^{\alpha 1} = R_{\alpha_1} \cdot \Delta O_2^{\alpha 1} \quad \text{or}$$
$$-\Delta BHCO_2^{\alpha 1} = 0.05 \times 10 = 0.5 \text{ vol. per cent}$$
$$-\Delta BHCO_2^{\alpha 4} = -\Delta BHCO_2^{\omega} - -\Delta BHCO_2^{\alpha 1} \quad \text{or}$$
$$-\Delta BHCO_2^{\alpha 4} = 3.2 - 0.5 = 2.7 \text{ vol. per cent}$$
$$\frac{-\Delta BHCO_2^{\alpha 4}}{\Delta O_2^{\alpha 4}} = R_{\alpha_4} = \frac{2.7}{20} = .135.$$

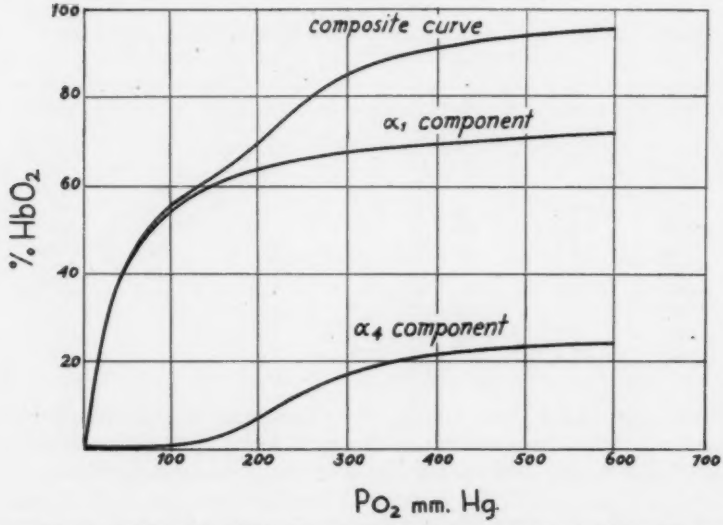

FIG. 3. Oxygen dissociation curves for the components of whole blood hemoglobin at 10 mm. CO_2-pressure. The upper curve represents the O_2-dissociation curve for the entire hemoglobin, obtained by adding the component curves together.

When there is no appreciable region where one component alone is contributing to the slope of the Haldane curve, an accurate determination of the R values is difficult or impossible and one must be satisfied with assumed values which will yield a theoretical curve closely fitting the experimental.

Having the R values, one is now in a position to construct a Haldane curve on the basis of the foregoing theory. With the hemoglobin fully

reduced, and the total CO_2 known under these conditions by extrapolating the experimental Haldane curve to 0 per cent O_2-saturation, one oxygenates the blood in steps of 10 per cent, determining for each step the amount of $BHCO_3$ released by each of the O_2-combining components. The total base released, $BHCO_3'$, subtracted from the

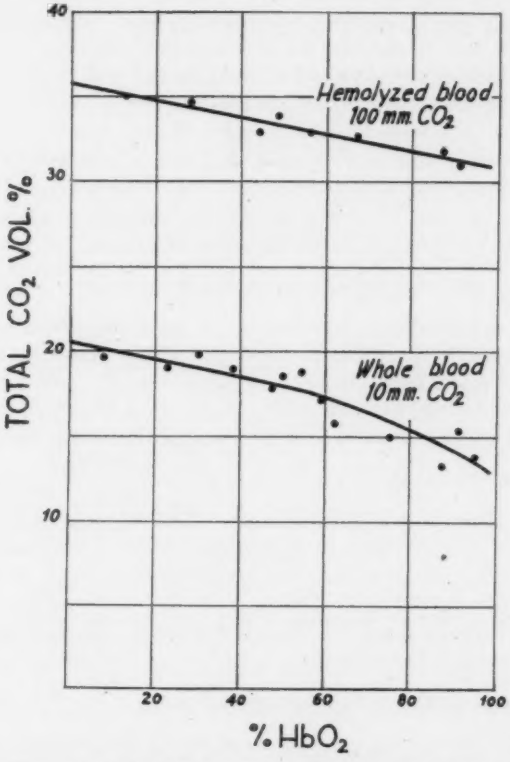

FIG. 4. Theoretical Haldane curves for whole blood at 10 mm. CO_2-pressure and hemolyzed blood at 100 mm. CO_2-pressure, drawn according to the theory discussed in the text. The points on the curves are those obtained by experiment.

total CO_2 present at the end of the preceding step in oxygenation will give a point on the Haldane curve for each increment of oxygenation. In Table I the data, derived in such a fashion, for the construction of a theoretical Haldane curve for whole tautog blood at 10 mm. CO_2 pressure are presented. The values in the first and last columns of this table have been used to plot the curve presented in Fig. 4, and the

points on the theoretical curve are those actually obtained by experiment. It is obvious that there is good agreement between theory and fact.

In the basic assumption for the interpretation of the anomalous Haldane curve for tautog blood it was pointed out that the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio for the entire hemoglobin *may be* inconstant. It has been demonstrated that such is the case for tautog whole blood at 10 mm. CO_2 pressure. However, it does not follow that the underlying theory can apply only to inconstant ratios, i.e. that the ratios *must* be inconstant at all times. A moment's consideration of equation (5) will make it clear that there could be such a set of R and ΔO_2 values for the components as to provide a practically constant release of base from the hemoglobin for each step in oxygenation. Should it so happen, for example, that the R values for the components are not too different, one might readily conclude experimentally that the $\frac{-\Delta BHCO_3}{\Delta O_2}$ ratio for the whole hemoglobin is constant—at least one would be tempted to draw a straight line through the experimental points. Or, what is more important, if it should so happen that the ΔO_2 values for each of the components remains practically constant over an extended range when the hemoglobin is oxygenated by equal steps, then it would follow, no matter what the R values, that the $\frac{-\Delta BHCO_3'}{\Delta O_2'}$ ratio would be practically constant in this same range. Whether such a circumstance would occur or not would be determined both by the values of the O_2-dissociation constants for the components and the shape of the O_2-dissociation curves they yield.

To illustrate the possibilities outlined above, we will consider the Haldane curve for hemolyzed tautog blood at 100 mm. CO_2 pressure. At this CO_2 tension experiment shows that the $\frac{-\Delta BHCO_3'}{\Delta O_2'}$ ratio is best represented as constant, yet analysis of the O_2-dissociation curve indicates that one is dealing with two components, α_1 and α_2. It is difficult to determine the exact values for R_{α_1} and R_{α_2} from the experimental Haldane curve since there happens to be no appreciable part of it where one component alone is acting. Such will be made clear by consulting the O_2-dissociation curves for the components presented in Fig. 5. It is evident that one must reduce the hemoglobin below 10 per cent O_2-saturation before there is any significant separation of the components. Since there is no apparent inflection in the Haldane curve even in this region, one must conclude that the R values are not

too different. The values we have finally taken are indicated in Table II. It is clear, furthermore, from Table II that the ΔO_2 values for the components remain about the same from 30 per cent O_2-saturation to 90 per cent for each 10 per cent step in oxygenation of the hemoglobin. Such a combination of factors can only mean that the theoretical Haldane curve will be practically a straight line, i.e. the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio is apparently constant. Figure 4 shows the theoretical curve for hemolyzed blood at 100 mm. CO_2-pressure drawn from the data of Table II. The points on the curve are those actually obtained

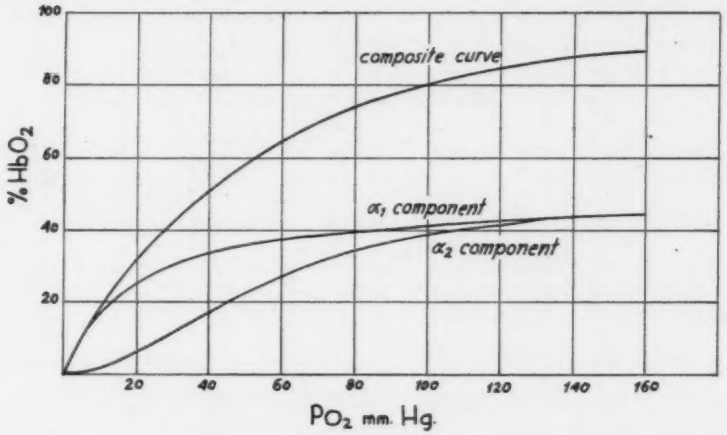

FIG. 5. Oxygen-dissociation curves for the components of hemolyzed blood hemoglobin at 100 mm. CO_2-pressure. The upper curve represents the O_2-dissociation curve for the entire hemoglobin, obtained by adding the component curves together.

by experiment. It must be concluded that under the right circumstances it is possible to have what appear to be constant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios even though more than one O_2-combining component is contributing to the oxygenation of the hemoglobin and the release of base. Such a state of affairs does not necessarily constitute an exception to the theory we have presented, but merely a special case.

Considering both the Haldane effect in whole blood at 10 mm. CO_2 pressure, where there is obviously an inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio, and the same effect in hemolyzed blood at 100 mm. CO_2 pressure, where

the ratio appears constant, it is clear that one must set forth certain qualifications concerning the type of ratio one might expect. If the values of R are quite different for each of the components and their equilibrium with oxygen is such as to yield widely varying ΔO_2 values for each step in the oxygenation of the hemoglobin (this would be dependent not only on the value of n but especially on the value of the O_2-dissociation constants for the components, which would have to be quite different in magnitude) then there should be no difficulty in demonstrating inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios. If, on the contrary, the R values for the components are closely similar, or especially if the components have such an equilibrium with oxygen as to provide nearly

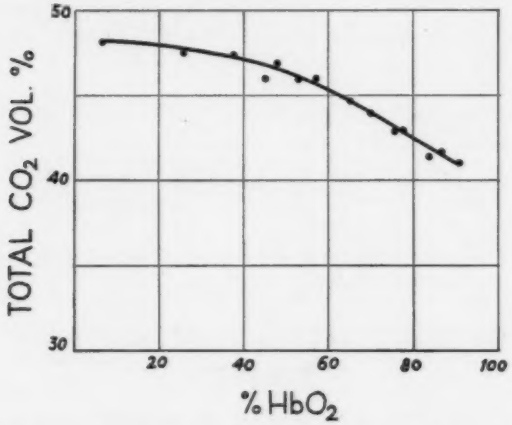

FIG. 6. Haldane curve for toadfish blood at pH 7.2. Data of Green and Root (1933)

constant ΔO_2 values for each step in the oxygenation of the hemoglobin (again this would be dependent on the value of n for the components, and their O_2-dissociation constants, which in this case would have to be more nearly alike) then the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratios would be practically constant, and experimentally would probably not show otherwise.

The inconstant $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio shown in whole tautog blood led us to re-examine some of the data of Green and Root (1933) on the blood of the toadfish. This blood is characterized by anomalous inflections in the O_2-dissociation curves adequately explained by the theory of components. At pH 7.2, for example, the O_2-dissociation

curve is satisfactorily described by assuming two O_2-combining components with widely different dissociation constants. Clearly, if the equilibrium with oxygen of these components at this pH is such as to provide anomalous inflections in the O_2-dissociation curve, then, if our theory is correct, the corresponding Haldane curve should present inflections, i.e. the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio should be inconstant. We have plotted the values of Green and Root for total CO_2 against the percentage of O_2-saturation and obtained the curve presented in Fig. 6. Although the slope of the curve is enhanced due to the fact that the CO_2 pressure was not kept constant (constant pH instead), it is evident from the inflection that the type of curve obtained is similar to that for whole tautog blood at 10 mm. CO_2 pressure, substantiating the theory we have presented.

There is a further matter of interest regarding the Haldane effect in the whole blood of the tautog. In our previous paper (Root and Irving, 1940), it was pointed out that when the CO_2-tension is raised sufficiently the Haldane effect tends to disappear, i.e. the ratio $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ approaches zero value. This happens when the hemoglobin has been partially inactivated and the remainder has been modified to a point where there is but a single O_2-combining component with a value of n equivalent to 1. The explanation probably is that if one decreased the pH sufficiently, the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio would diminish for a particular hemoglobin, even if the components themselves did not change their behavior, for one might eventually reach a point where the titration curves for the reduced and oxygenated forms of the hemoglobin are converging toward each other. In other words, it is not safe to assume in any situation where CO_2 modifies the behavior of hemoglobin that the change in slope of the Haldane curve is entirely due to this effect of CO_2. It will hold only as long as the pH of the blood remains in the region where the titration curves for reduced and oxygenated hemoglobin parallel each other. Outside these limits the $\frac{-\Delta BHCO_3^{\omega}}{\Delta O_2^{\omega}}$ ratio will change in a manner quite independent of any modification of the hemoglobin or its O_2-combining components.

It can be seen from the major part of the foregoing discussion that the argument for the peculiarities of the Haldane effect in tautog blood rests primarily on the theory that the hemoglobin is made up of different O_2-combining components. One might turn the argument around and say that the peculiarities of the Haldane effect offer strong

support for the theory of components; for it is difficult to see how one could get inflected Haldane curves, such as we have found in whole blood, without having different O_2-combining components present, each of which independently affects the CO_2-combining power of blood on oxygenation. As the situation now stands it has been shown that a single scheme can be used to describe both the effect of CO_2 on the oxygenation of hemoglobin and the reciprocal effect of oxygenation on CO_2-combining power.

The authors wish to acknowledge their indebtedness to Virginia Safford and Henry Brown for technical assistance, and to Dr. Paul S. Galtsoff, Director, and Mr. Robert Goffin, Superintendent of the U. S. Bureau of Fisheries at Woods Hole for their coöperation during this investigation. They also wish to express to Professor A. C. Redfield their appreciation for his suggestions and criticisms in the preparation of the manuscript.

Summary

1. A detailed study has been made of the effect of CO_2 on the equilibrium between hemoglobin and oxygen in whole and hemolyzed blood of the tautog.

2. The study of the O_2-dissociation curves of whole blood has shown that the addition of CO_2 up to 100 mm. pressure changes the shape of the curves from sigmoid to rectangular hyperbolae with approximately 50 per cent of the hemoglobin inactivated. The intermediate stages in the transformation produce complex dissociation curves which can be described by assuming that fish hemoglobin is made up of different O_2-combining components acting independently of each other and combining with different amounts of oxygen at a time.

3. Hemolysis renders the hemoglobin less sensitive to CO_2 as evidenced by the fact that the O_2-dissociation curves move far to the left of those for whole blood; that the O_2-combining components which combine with more than one molecule of O_2 at a time show greater stability than they do in whole blood as the CO_2 tension is raised; and that there is no hemoglobin inactivation up to at least 100 mm. CO_2. There is still a prominent Bohr effect, however, and the O_2-combining components still gradually change their behavior as the CO_2 tension is raised.

4. Based primarily upon the characteristics of the equilibrium between hemoglobin and oxygen, a theory is offered to explain certain peculiarities of the effect of oxygenation on the CO_2-combining power of the blood (Haldane effect). The theory offered provides a common

explanation for the anomalies in the effect of CO_2 on oxygenation of the hemoglobin and in the reciprocal effect of oxygenation on the CO_2-combining power of the blood.

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OBSERVATIONS ON THE FOOD HABITS OF ENTAMOEBA MURIS AND ENTAMOEBA RANARUM

D. H. WENRICH

(From the Zoölogical Laboratory, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole, Mass.)

I. Introduction

While studying nuclear structure and nuclear division in *Entamoeba muris* (Wenrich, 1940), casual observations indicated that there were food preferences among different individuals and among different populations of these amoebae. Further investigation showed that these amoebae often developed temporarily differentiated ingestion tubes which stain intensely with Heidenhain's haematoxylin. A preliminary report on these observations was made at the Marine Biological Laboratory and an abstract published (Wenrich, 1939). Subsequent studies extended the survey to other sets of slides showing *E. muris* from both rats and mice and similar conditions were then discovered for *E. ranarum* from frogs and toads. The present more extended and illustrated report covers the entire set of observations.

These studies have been made partly at the Marine Biological Laboratory and partly at the University of Pennsylvania. They have been made entirely on fixed and stained slides. A variety of fixing and staining agents have been employed in preparing the slides from caecal and rectal contents but the majority of the smears have been fixed in Schaudinn's sublimate-alcohol-acetic and stained with Heidenhain's haematoxylin.

The rats and mice from which slides were made were secured from a variety of sources but a good many rats were obtained from the Wistar Institute and to The Institute, and especially to Doctor Helen Dean King, grateful acknowledgment is made. Acknowledgment is also made to the staff of the Department of Zoölogy of the University of California at Berkeley, for aid in securing frogs and tadpoles and for facilities for their examination. Most of the observations on *Entamoeba ranarum* were made on material from *Rana pipiens* examined at the University of Pennsylvania.

Observations

Entamoeba muris

Entamoeba muris lives in the caecum of rats and mice. Of the more than 500 rats and more than 100 mice that have been examined during the past twenty-five years, relatively few have had amoebae in sufficient numbers to warrant detailed study.

The more striking results of this study of the food habits of *E. muris* are: (1) that there is a great diversity in the kinds of objects selected as food; (2) that some individuals may limit themselves, for a time at least, to a single type of food with which they may engorge themselves, while others may engulf a considerable variety of food objects; (3) that certain populations of amoebae, that is, those from a single host rat or mouse, may show strong tendencies to select one kind of food material, while in other populations, such tendencies are not manifested; and (4) that differentiated ingestion tubes are developed for the ingestion of some kinds of food.

The food objects fall into two natural divisions or groups; those of a plant nature and those of an animal nature. The former group is represented by a great variety of bacterial organisms, a few of which are illustrated by Figs. 1 to 4 and 28, yeasts (Fig. 5), *Blastocystis* (Fig. 12), plant filaments (Figs. 6, 24–27), all of which are apparently resident within the caecum; and starch grains (Figs. 7, 13, 15–18) from the host's diet. Animals are represented by the other Protozoa resident in the host's caecum and small intestine and by various types of cells derived from the host. Among the Protozoa are the trichomonads (Figs. 11, 14, 29–32), *Chilomastix* and *Hexamitus pulcher* from the caecum; and *Giardia* (Figs. 10, 13) and *Hexamitus muris* which come down from the small intestine. Host cells found ingested were erythrocytes (Fig. 8), leucocytes (Figs. 9, 19), and epithelial cells.

Diversity of food preferences among individuals of a population as well as instances of specialization by individuals are illustrated by Figs. 3, 6, 7, and 10, all from the caecum of one mouse. Figures 15, 17, 18, 20–27 are also from the same population. Preferences by individuals are illustrated on Plate I, where each amoeba has filled its cytoplasm with one kind of food. Figures 1 to 7 show ingested plant materials so that these individuals might be considered to have been "herbivorous," at least temporarily. Figures 8 to 11 illustrate individuals which were "carnivorous" at the time that they were killed, and the amoebae in Figs. 13 and 14 could be called "omnivorous" since they contain food objects of both plant and animal nature.

Population food preferences are occasionally noteworthy. The fusi-

form bacillus shown in Fig. 1 is probably the most common food object seen and many populations show a high percentage of their members containing this organism. The colonial species seen in Fig. 2 is sometimes given preference by a population. On the set of slides from which this figure was drawn, about 65 per cent of the amoebae had ingested one or more of these colonies, most of which, however, were much smaller than the one shown in Fig. 2 (cf. Fig. 14). Two quite different types of diplococcoid species are shown in Figs. 3 and 4. These are not uncommon food inclusions, but it is unusual to see so many of either kind in any single individual. Many other types of bacteria are found in the amoebae, but they have not been identified or drawn.

Yeasts are not uncommon food objects, but specialization on yeasts, as shown in Fig. 5, is uncommon. Several populations were found in which ingested filaments were more than occasionally seen, although the proportion of individuals enclosing filaments in any one population was never more than 2 or 3 per cent. Starch grains were not very commonly seen, although a number of populations included individuals which had ingested such grains.

Populations with ingested host cells were uncommon. Epithelial cells inside amoebae were seen only on a few occasions. Erythrocytes taken as food were noted in only two populations which were from mice. In one the number of individuals showing erythrocytes was greater than in the other, but in both there was a tendency for the same individual to ingest several red cells, as illustrated by Fig. 8. Ingestion of leucocytes was also uncommon and the one population in which a number of

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PLATE I

Figs. 1–11 showing examples of specialization by individual amoebae. Figs. 3, 6, 7, and 10 are from the same mouse. Figures 8 and 9 are also from mice. Figures 1, 2, 4, 5, and 11 are from rats.

Figs. 1–7. Examples of "herbivorous" food preferences.

Fig. 1. Amoeba filled with fusiform bacillus—the most common type of food.

Fig. 2. Amoeba containing a large colonial organism.

Fig. 3. Amoeba containing many small diplococcoid bacteria.

Fig. 4. Amoeba with large diplococcoid species.

Fig. 5. Amoeba with a dozen yeast cells.

Fig. 6. Amoeba with long coiled filament.

Fig. 7. Amoeba containing six starch grains. Note deeply-stained granules on side of two of them.

Figs. 8–11. Examples showing "carnivorous" habits.

Fig. 8. Amoeba showing five erythrocytes; one more was under the nucleus.

Fig. 9. Amoeba with four leucocytes.

Fig. 10. Amoeba with three specimens of *Giardia*.

Fig. 11. Amoeba with eight specimens of *Trichomonas muris*.

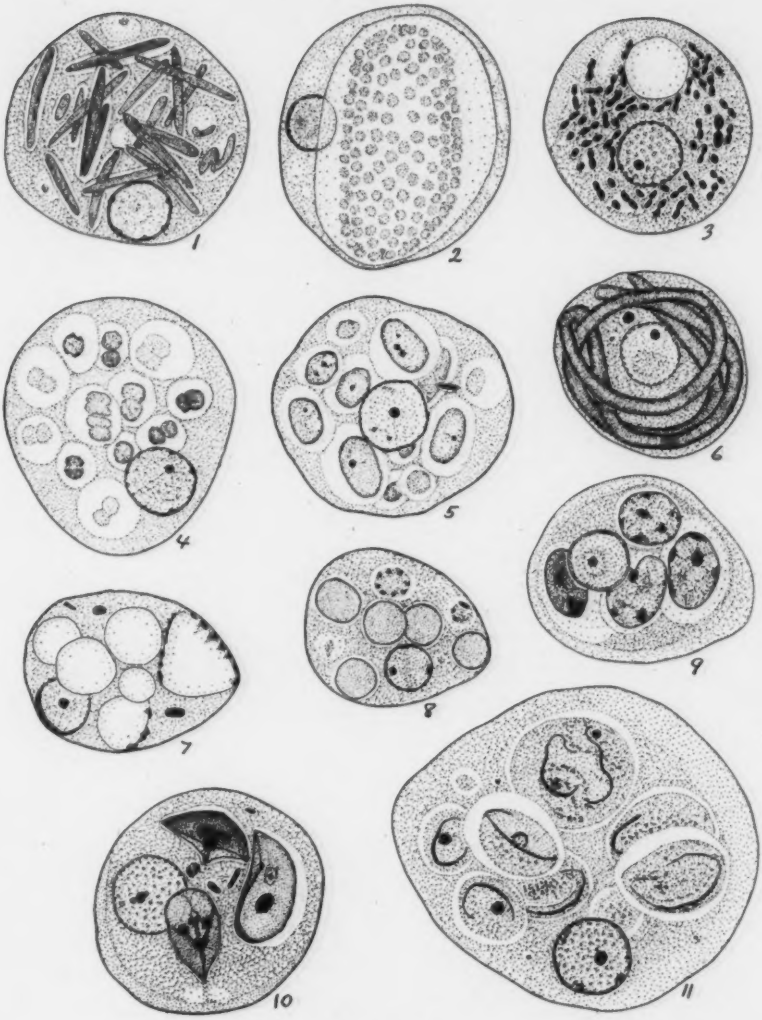

PLATE I

All figures are from fixed and stained slides. They have been drawn with the aid of a camera lucida at an initial magnification of × 3000 and reduced about one-third in printing. Figures 1–33 are of *Entamoeba muris* and Figs. 34–42 are of *E. ranarum*.

amoebae were found with ingested leucocytes was in a mouse. Individual preference is illustrated in Fig. 9.

Among the ingested Protozoa, *Trichomonas muris* was the most common. In many populations it was rarely seen as a food object, while in others it was the population preference. In one count from a slide from a rat about 80 per cent showed one or more trichomonads in various stages of digestion. Individual preferences for this flagellate to the exclusion of other food objects were common in such populations. *Chilomastix bettencourti, Hexamitus pulcher* and *Hexamitus muris* were seen within the amoebae on only a few occasions. *Giardia* was seen more frequently but was not commonly observed. In the population from a mouse, from which Fig. 10 was taken, between 3 and 4 per cent showed one to three individuals of this flagellate. In this population only the trophic stages of *Giardia* were ingested, although the cysts were available.

Ingestion Methods.—Entamoeba muris apparently adopts somewhat different methods for the intake of food, depending upon the nature of the material to be ingested. In the case of starch, it appears from Figs. 15 to 18 that food cups are formed which are just big enough to take in the granules with no vacuolar space between the food body and the cytoplasm. The absence of a vacuole around starch grains is also indicated in Figs. 7 and 13. In some cases (Figs. 7 and 13), deeply-stained bodies are seen in the cytoplasm which is in contact with the starch grain. These bodies are absent in other cases (Figs. 15–18) and in Fig. 7 only

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PLATE II

Figs. 12, 14, from rat. Figs. 13, 15, 17, 18, 20 and 21–33 from one mouse. Figs. 16 and 19 from another mouse.

FIG. 12. Amoeba with specimen of *Blastocystis*.

FIG. 13. Amoeba containing two starch grains, a specimen of *Giardia* and several bacteria.

FIG. 14. Amoeba containing *T. muris*, six colonial organisms and two bacilli.

FIG. 15. Amoeba with starch grain half ingested. Note that edge of food cup and cytoplasmic layer in contact with starch are deeply stained.

FIG. 16. Amoeba containing large starch grain.

FIGS. 17 AND 18. Show ingestion of starch grain almost completed. Note deeply-stained edges of closing-in pseudopodia.

FIG. 19. Amoeba with two partly ingested leucocytes. Note constriction of leucocytes.

FIG. 20. Amoeba with empty food cup. Wall of cup composed of denser cytoplasm but not deeply stained.

FIG. 21. Amoeba with empty food cup. Wall of cup deeply stained.

FIG. 22. Amoeba with food cup turned "wrong-side-out."

FIG. 23. Amoeba with food cup with partly ingested food object and deeply-stained walls.

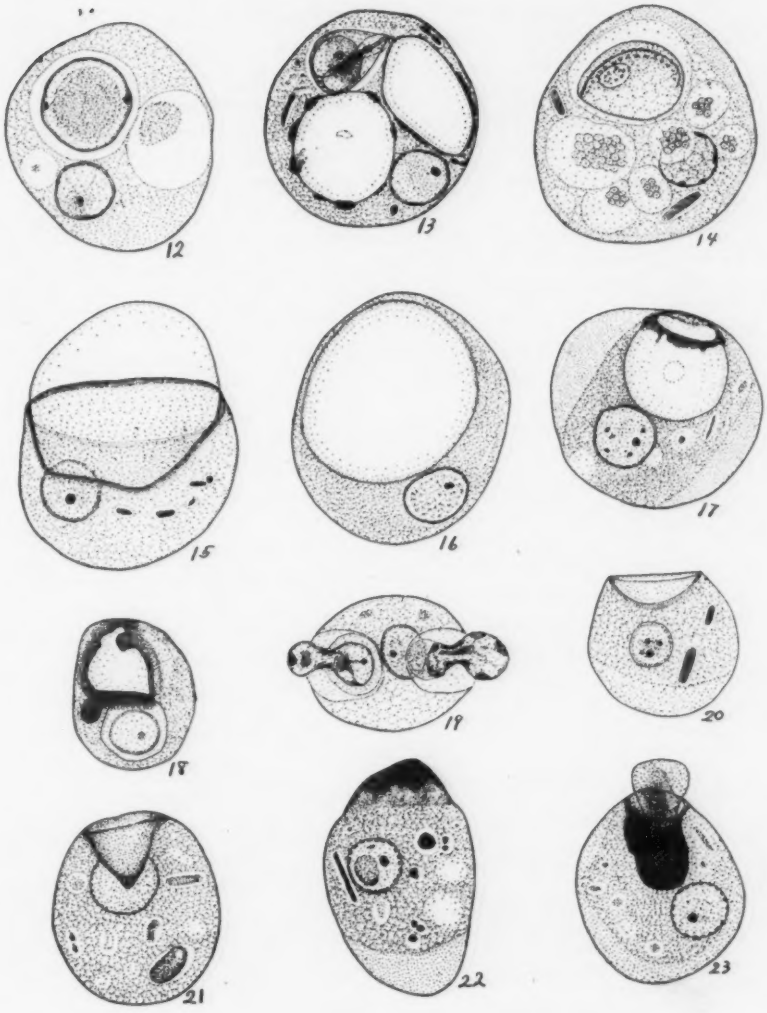

PLATE II

two of the six grains show them. Their nature is problematical, but it is assumed that they are related to digestion. In a number of cases the edges of the advancing pseudopodia which were closing in on a starch grain were deeply stained (Fig. 15) and more especially during the later stages of the enclosing process (Figs. 17 and 18).

In a number of instances, empty food cups were seen (Figs. 20 and 21) and the walls of these cups were obviously composed of denser cytoplasm which might (Fig. 21) or might not (Fig. 20) stain intensely. The condition shown in Fig. 22 is interpreted to be a food cup turned "wrong-side-out." In Fig. 23 a food cup is shown with a partially ingested object and with deeply-stained walls. The middle part of this tube-like cup is more deeply stained than the rest, suggesting greater thickness or greater density.

Figure 19 shows a small specimen of *E. muris* which was fixed while ingesting simultaneously two leucocytes, one at each side. Constriction of the leucocytes is indicated, but the edges of the two food cups are not deeply stained.

Ingestion of filaments was in some cases (Figs. 24 and 27), but not in others (Figs. 25 and 26), accompanied by the formation of deeply-stained ingestion tubes in connection with the ingestion cones. In Fig. 25 a filament has been surrounded at a region away from either end. Ingestion cones were formed and were advancing along the filament in both directions. In Fig. 27, an especially long, deeply-stained food tube is shown. From the left end of this tube and proceeding toward the right, there are three thickenings in the wall of the tube on alternate sides, suggesting a spiral band of more intensely staining material.

A definite "mouth" or ingestion cone and deeply staining "pharynx"

PLATE III

Figs. 24–27 from same mouse. Fig. 30 from another mouse. Figs. 28, 29, 31–33 from rats.

FIG. 24. Amoeba with long filament partly coiled inside. Note deeply-stained ingestion cone and "pharynx."

FIGS. 25 AND 26. Amoeba with partly ingested filaments; ingestion cones not deeply stained.

FIG. 27. Amoeba with partly ingested filament; long, deeply-stained "pharynx."

FIG. 28. Differentiated "mouth" and "pharynx" with partly ingested bacillus.

FIG. 29. Amoeba with ingestion of *T. muris* almost completed. Note deeply-stained "mouth" followed by undifferentiated food cavity with deeply-stained constriction farther in.

FIG. 30. Amoeba ingesting *T. muris* through differentiated "pharynx."

FIGS. 31 AND 32. Amoebae with apparently broken ingestion tubes, due to traumatism. In Fig. 32 the lower tube is all inside and is possibly a constriction tube.

FIG. 33. Amoeba with internal constriction tube.

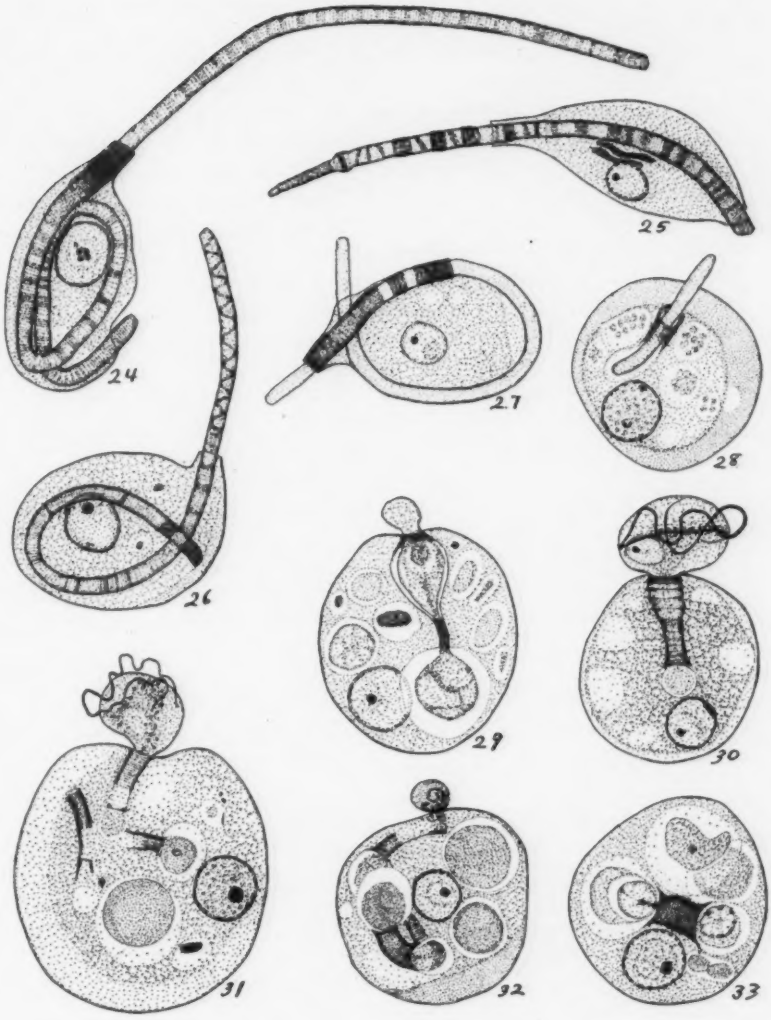

PLATE III

for the ingestion of rod-shaped bacteria are shown in Fig. 28. A number of narrow tubes of this type were seen containing partly ingested bacterial rods.

Trichomonas is apparently ingested by differentiated tubes. A typical case is illustrated in Fig. 30 and many variations of this picture have been seen. In one case the axostyle had been drawn into such a tube while the remainder of the victim remained outside. In another case the anterior flagella had been taken in and the prey had descended a short distance "head first." In still another case the posterior flagellum had been ingested ahead of the rest of the animal. Apparently the amoeba is able to "seize" the flagellate at any point on the latter's surface. In one instance two converging tubes were attached to one trichomonad. The ingestion tube varies in length, up to more than half the width of the amoeba. In some cases the diameter varies in different regions (Figs. 29 and 30). In Figs. 31 and 32 traumatic fragmentation of ingestion tubes is indicated. The amoeba illustrated by Fig. 31 showed definite signs of injury. In a few cases trichomonads were found partly incased in broad food cups, such as shown in Figs. 21 and 23. It is possible that early stages of ingestion may involve such food cups, to be followed by the gradual development of the differentiated tubes such as seen in Fig. 30.

There is evidence that similar tubes are employed to break up food masses, as illustrated in Fig. 33. It is possible that in Fig. 32 a combination of an ingestion tube and a constriction tube is indicated.

Entamoeba ranarum

The finding of the conditions just described for *Entamoeba muris* led to an examination of smears made from the rectum of frogs and

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PLATE IV

All figures of Entamoebae from frogs. Figs. 34, 35, 36, 38, 39, and 40 of *E. ranarum* from *Rana pipiens*. Figs. 37, 41, 42, of possibly different species, from California frog, *Rana draytonii*.

FIG. 34. Amoeba with a dozen specimens of *Hexamitus*.

FIG. 35. Amoeba with a specimen of *Trichomonas augusta*.

FIG. 36. Amoeba with four specimens of *Chilomastix*.

FIG. 37. Amoeba with five host cells.

FIG. 38. Amoeba with partly ingested filament; two deeply-stained "pharyngeal" regions.

FIG. 39. Amoeba with partly ingested short filaments; not deeply-stained "pharynx."

FIG. 40. Amoeba containing one host cell nucleus and a partly ingested second host cell nucleus.

FIG. 41. Amoeba with empty food cup. (cf. Figs. 20, 21.)

FIG. 42. Amoeba showing constriction of food inside cytoplasm.

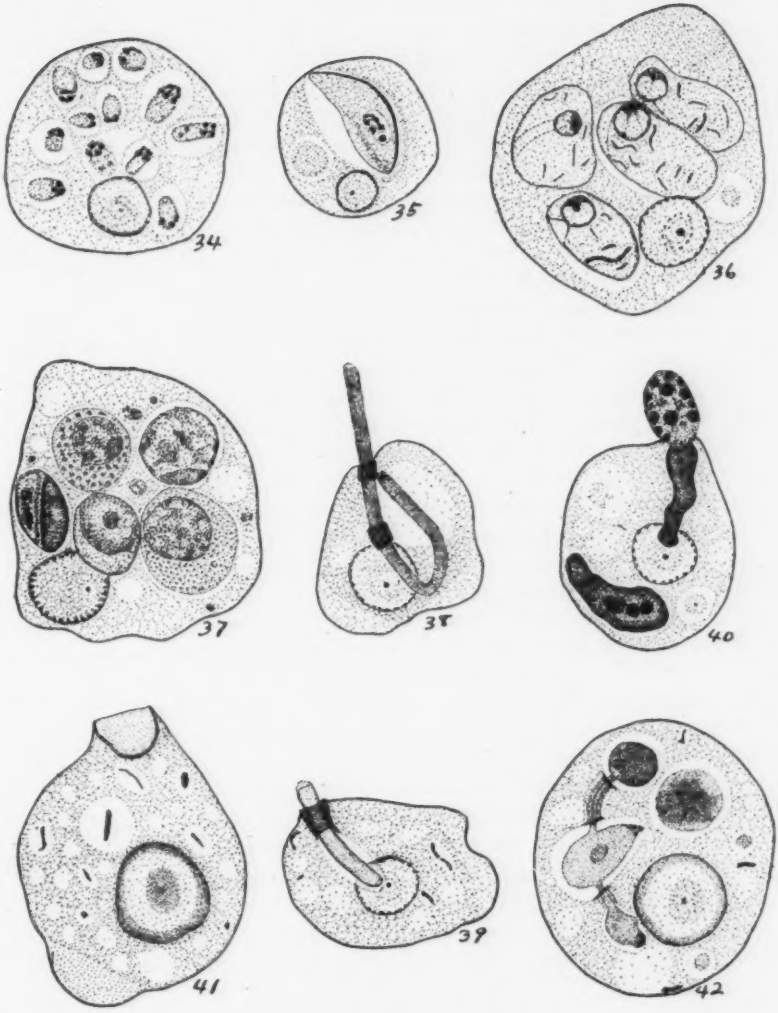

PLATE IV

toads. Here similar conditions were observed for *E. ranarum*. Some of these conditions are illustrated in Figs. 34–42. Individual specialization is indicated by Figs. 34–37. The amoeba in Fig. 34 contains twelve specimens of *Hexamitus*. The population in this case tended to favor *Hexamitus* as a diet, since 17 per cent had ingested one or more individuals. On the same slide 3 per cent of the amoebae contained trichomonads. In a count on a slide showing another population, 50 per cent of the amoebae contained *Hexamitus* as food. The population on this other slide showed diversity of choice, however, as indicated by the ingestion of filaments (Figs. 38 and 39) and host cell nuclei (Fig. 40). In another frog, *Chilomastix* was favored by a considerable number of the amoebae (Fig. 36).

On slides from the California frog, *Rana draytonii*, a large majority of the amoebae contained host cells, apparently leucocytes, although some may have been erythrocytes (Fig. 37). As many as twelve such cells were counted in a single amoeba. A more balanced diet was represented by an amoeba with four host cells and four individuals of the plant, *Blastocystis*. (The amoebae from this species of frog have a nuclear structure considerably different from that typical of *E. ranarum*, and may therefore be a different species.) In a similar amoeba from a California tadpole, a starch grain was seen.

The methods of ingestion employed by these amoebae from frogs and toads are apparently the same as those employed by *E. muris*. In the ingestion of filaments, the formation of ingestion tubes with deeply-stained annular thickenings is shown in Fig. 38. A short differentiated "pharynx" is shown in Fig. 39. Figure 41 shows an empty food cup similar to that seen in *E. muris* (cf. Figs. 20, 21). Constrictions for the breaking up of food masses are shown in Fig. 42, where there are two constrictions being applied simultaneously to a single food body. An internal constriction tube similar to that shown in Fig. 33 for *E. muris* was also seen on the same slide as that from which Fig. 42 was taken. Altogether, the food habits of *E. ranarum* are quite parallel to those of *E. muris*.

Discussion

Most of the extensive literature dealing with the feeding activities of amoebae is concerned with free-living species, there being relatively few reports on the food habits of those that are parasitic. Since the present study has been limited to fixed and stained specimens, the behavior aspects must be inferred, and an extensive discussion of amoeboid nutrition would be inappropriate. However, some interesting interpretations

can be made and their relation to existing literature can be noted. The following items seem worthy of attention here: (1) the diversity of food objects ingested; (2) preferences of individuals and populations for certain kinds of food; (3) methods of ingestion; (4) the breaking up of food bodies after their ingestion; and (5) the appearance of secretion bodies in contact with ingested starch grains.

Diversity of Food Materials Ingested.—Most of those who have studied *Entamoeba muris* have remarked upon the variety of food objects in the cytoplasm of the amoebae. This diversity has been compared to that frequently mentioned for *E. coli* from man. In amoebae from the caecum of mice, Wenyon (1907) noted bacteria of various kinds, *Trichomonas, Giardia, Hexamitus* and its cysts, yeast cells, and epithelial cells. Kessel (1924) noted the inclusion of *Chilomastix* and smaller amoebae besides different kinds of bacteria, and Wang and Nie (1934) state that ingested food consists mainly of starch grains, intestinal bacteria and plant debris. To these lists the present study adds *Blastocystis,* long plant filaments, and host erythrocytes and leucocytes.

A similar diversity of food inclusions in *E. ranarum* was noted in the present study. Dobell (1909) made few comments on the food of this species but remarked that when blood corpuscles and broken-up epithelial cells were available in the large intestine the amoebae readily ingested them. In the present study host cells, not clearly identifiable, but possibly including both erythrocytes and leucocytes, were conspicuous food objects in the amoebae from *Rana draytonii.*

Individual and Population Food Preferences.—The tendency for a single amoeba to ingest repeatedly the same kind of food object is well known for *E. histolytica,* individuals of which may contain as many as thirty to forty erythrocytes at one time; and, in cultures, these amoebae may engorge themselves with starch grains. Frye and Meleney (1936) noted that in cultures this species varied considerably in its tendency to ingest erythrocytes, depending upon the conditions in the medium with which the amoebae were surrounded. Pavlova (1938) has confirmed some of these results and states further that the capacity of *E. histolytica* to ingest red cells depends upon the pH of the medium, the capacity being greatest at pH values between 5.6 and 6.5. Semenoff (1938) reported that ingestion of erythrocytes did not take place unless the latter adhered to the surface of the amoebae. It is reasonable to suppose that *E. muris* and *E. ranarum* capture bacteria and active flagellates by an initial adhesion of the latter to the surface of the amoebae, and that this adhesion would, in turn, be controlled by various external and internal factors. One wonders if such factors would be sufficiently limited or specific in their effects to explain repeated ingestion of one kind of food

body by an individual amoeba. If this were so, then the preference of a large percentage of some populations for one kind of food might be similarly explained. However, the divergence of choice commonly exhibited within a population would indicate that individuals tend to vary among themselves as to their physiological state. It is doubtful if population preferences represent racial, that is, genetic, differences, although such a possibility cannot be ignored.

Methods of Ingestion.—Much has been written about the methods by which amoebae take food into their bodies, but Ivanić (1933) was apparently the first to call particular attention to the formation of a "cytostome" and accompanying tube sufficiently differentiated to stain deeply with iron hematoxylin. He first noted such structures in *Amoeba vespertilio, Amoeba iuvenalis* and an unnamed species of *Hartmanella,* but extended the observations to *Amoeba entzi* (1936), *Hartmanella maasi* (1936*a*) and *H. blattae* (1937). I have also seen a deeply-stained (iron hematoxylin) ingestion apparatus in two different small free-living amoebae of the *Hartmanella* type, where the "cytostome" was funnel-shaped, the funnel opening outwardly. On one slide showing these amoebae nearly every individual displayed from one to a dozen of these funnels at various points on the periphery.

In *E. muris,* ingestion methods seem to be much more diversified, apparently adapted to different kinds of food, but they include the formation of specialized tubes which stain intensely with iron hematoxylin.

In many of his illustrations Ivanić shows, proximal to the "cytostome," capacious vacuoles and speaks of food bodies as being drawn into them. Some of the conditions seen in the present study would lend support to this interpretation. In Fig. 19 the concentration of the more fluid cytoplasm at the inner ends of the partly ingested leucocytes together with the constriction at the "mouth" suggests suction. Suction is also suggested in the ingestion of *Trichomonas* by *E. muris;* here various portions, anterior flagella, posterior flagellum, etc., can be identified as having gone down the "pharynx" in advance of other portions, and often a rounded globule of trichomonas protoplasm occupies an internal vacuole while other portions of the flagellate remain outside and the two parts remain connected through the tube-like "pharynx" (Figs. 29, 30). The enlarged vacuoles into which the short filaments are entering in Figs. 28 and 39 suggest the same thing. Semenoff (1937, 1938) found that *E. histolytica* frequently extracted the nuclei from frog and chick erythrocytes although sometimes ingested fragments might include some cytoplasm. It is difficult to understand how suction

can be developed within an amoeboid cell, but the evidence at hand favors that interpretation.

The ingestion of starch appears to take place by simple extension of pseudopodia over the food object and in contact with it (Figs. 15, 17, 18). This method resembles that frequently reported for free-living amoebae during the act of ingesting starch or other solid bodies. Brug (1928) saw a living specimen of *E. histolytica* enter a group of starch grains and emerge two or three minutes later with four larger and two smaller grains in its cytoplasm, but he did not see the method of intake.

E. muris and *E. ranarum* apparently ingest filaments in a manner similar to that described for free-living species in such classical papers as those of Leidy (1879) and Rhumbler (1898); and more recently Comandon and Fonbrune (1936), have recorded their observations with motion pictures. Ivanić (1933) showed that ingestion of filaments by *A. vespertilio* is accompanied by deeply stainable thickenings along the ingestion tube and the present study reveals similar conditions for *E. muris* and *E. ranarum* (Figs. 24, 27, 38). That a differentiated tube, such as shown in Fig. 27, is fairly stable—for a time at least—is indicated by the finding of a similar tube attached to a bent filament but with the remainder of the amoeba missing—probably having been torn off during the smearing process. It is probable that Fig. 25 represents an early stage in the bending of the filament, a process which might well result in the condition seen in Fig. 27. It is interesting that Figs. 25 and 26 do not show the deeply-stained walls of the ingestion tube that are seen in Figs. 24 and 27. It is doubtful if these differences are the result of variations in the destaining process, since Figs. 24 and 26 were drawn from the same slide. Stainability seems to vary with density of protoplasm and the density is doubtless correlated with degree of contraction.

Peristaltic contractions may be indicated by the successive thickenings on alternate sides of the "pharynx" shown in Fig. 27. Comandon and Fonbrune (1936), employing motion pictures, record the observation of waves of contraction along the ingestion cone surrounding a filament in *A. verrucosa.* Peristaltic action during ingestion of trichomonads by *E. muris* may also be indicated by the differences in diameter of the "pharynx" shown in Figs. 29 and 30.

The Breaking Up of Food Bodies After Their Ingestion.—The ability of amoebae to break up food masses into smaller units has been noted by a number of observers, for example by Leidy (1879) and Penard (1912). More recently Entz (1925) has provided a good description of successive constrictions of food objects as seen in *Amoeba vespertilio;* and, in a later paper (1932) he reviewed the literature show-

ing instances of the breaking up of food masses in both amoebae and ciliates, and also in the flagellate, *Collodictyon*. Ivanić (1936) described the constriction of ingested food masses, sometimes several such constrictions taking place simultaneously; and Mast (1938) reported the breaking up of ingested *Colpidium* in the cytoplasm of *Amoeba proteus*.

The present record seems to be the first for the breaking up of food in a species of *Entamoeba* and none of the observers referred to above have reported the presence of deeply-stained constriction tubes, such as shown in Figs. 33 and 42. It is possible that the deeply-stained tube in Fig. 33 represents a "pharynx" which has persisted after the prey was ingested, although the vacuole at each end does not suggest that interpretation; and such an interpretation would not be applicable to the condition seen in Fig. 42.

Wenyon (1907) speaks of seeing several specimens of *Trichomonas* in a single vacuole in *E. muris* (see his Fig. 1). On the slides used in the present study, flagellates, or their fragments, were almost always in segregated vacuoles. However, large vacuoles, each containing many bacteria, were sometimes seen and one wonders if fusion of vacuoles may take place as well as their subdivision. Ivanić, however, believed that a succession of objects would be taken in through a single "cytostome."

Digestive Granules in Contact with Ingested Starch Grains.—Figure 13 shows a specimen of *Entamoeba muris* containing two starch grains, each of which has deeply-stained masses at its periphery. Figure 7 shows an amoeba with six starch grains and similar stained bodies are seen at the sides of two of them. That it takes some time for such bodies to appear is indicated by their absence in Figs. 15 to 18 where starch grains are being ingested, and also their absence from four of the six grains in Fig. 7. It seems reasonable to assume that these bodies in contact with food represent secreted material having a digestive function. Very similar bodies are shown by MacLennan (1936) for food bodies in *Ichthyophthirius* and he identifies them as elements of the vacuome since they react positively to neutral red and to the Kolatchev-Nassanov method for impregnation of Golgi material. Volkonsky (1934) shows similar neutral red staining bodies outside starch grains ingested by a large granulocyte of *Phascolosoma*, and also by a choanocyte of *Clathrina coriacea*. In his general review of cytoplasmic inclusions in Protozoa, MacLennan (1941) refers to such bodies as digestive granules.

Various observers have denied to free-living amoebae the capacity to digest starch. However, the avidity with which *E. histolytica* and other endamoebae ingest this form of carbohydrate is well established. It

should not be surprising therefore, if, as in the other cells referred to, digestive secretions should be elaborated by such amoebae for the digestion of starch.

Summary

On the basis of observations on fixed and stained slides showing *Entamoeba muris* and *E. ranarum*, the following observations and interpretations have been made.

In general, these species of *Entamoeba* show great diversity in the kinds of food ingested. *E. muris* more commonly feeds on a fusiform bacillus but its diet includes many other types of bacteria, *Blastocystis*, yeasts, plant filaments, starch grains, *Trichomonas, Chilomastix, Hexamitus*, and host erythrocytes, leucocytes and epithelial cells. *E. ranarum* shows a similar range of food objects.

Individuals often select for a time, at least,—a single kind of food, with which they may engorge themselves. Others are more omnivorous in their selection.

Populations from a single host may show decided preferences for one type of food; for example, about 80 per cent of one population of *E. muris* contained one or more specimens of *Trichomonas*.

A diversity of methods of ingestion is indicated. Starch grains are surrounded by enveloping pseudopodia without the formation of a fluid-containing vacuole around them. Trichomonads appear to be drawn through an ingestion tube with walls sufficiently differentiated to stain heavily with iron hematoxylin. Plant filaments are taken in through similar tubes some of which show the deeply-stained walls. There is evidence that differentiated tubes are employed to constrict food bodies into smaller units.

Bodies which stain with iron hematoxylin have been seen in contact with ingested starch grains in *E. muris*. These are interpreted as digestive granules in the sense that this term is used in the review by MacLennan (1941).

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STUDIES ON THE GROWTH OF INTEGUMENTARY PIGMENT IN THE LOWER VERTEBRATES

I. The Origin of Artificially Developed Melanophores on the Normally Unpigmented Ventral Surface of the Summer Flounder (Paralichthys dentatus) [1]

CLINTON M. OSBORN

(From the Department of Anatomy, the Ohio State University, and the Woods Hole Oceanographic Institution, Woods Hole, Mass.)

Considerable evidence has accumulated to indicate that melanophores may be grown experimentally on certain fishes and amphibians in areas where these cells fail to develop naturally. Cunningham (1891, 1893, and 1895), working with several species of flatfishes; von Frisch (1911), using *Esox* and *Nemadulus;* and Osborn (1940*a*, *b*, and *c*), studying the summer flounder (*Paralichthys dentatus*) and the common bullhead (*Ameiurus melas*) have all reported success in growing melanophores on the normally unpigmented ventral [2] surfaces of these teleosts. Experimenting with the urodele, *Salamandra maculosa,* Herbst and Ascher (1927) were able to develop abnormal amounts of pigment ventrally. In spite of these observations the origin of the newly developed melanophores has remained an open question. Alternative possibilities are obvious: either they differentiate *in situ* or they migrate in from other areas.

This paper brings together the results of experiments referred to in an earlier report (Osborn, 1940*a*) designed to gain more information concerning the source of experimentally developed melanophores.

Materials and Methods

A freshly caught stock of adult flounders 15 to 18 inches long was maintained to avoid abnormal conditions in pigmentation which sometimes arise from prolonged sojourns in unnatural laboratory surroundings. The desired amount of pigmentation was developed ventrally in

[1] Contribution No. 296 of the Woods Hole Oceanographic Institution, whose research facilities and financial aid provided for this investigation are genuinely appreciated.

[2] The term "ventral" will be used to refer to the lower normally unpigmented surface of the animal. In the flatfishes the unpigmented side is more strictly the right or the left side, depending upon the species. In the summer flounder the right side is white.

an apparatus similar to that previously described (Osborn, 1940*a*) with minor improvements. When a fish was sacrificed, at least 40 scales plucked from widely separated areas on the ventral surface were fixed in 5 per cent neutral formalin. Of these, ten were dehydrated in alcohol, cleared in xylol and mounted in Clarite; ten were mounted in glycerine jelly directly following fixation; and 20 were treated according to Laidlaw's modification (1932*a*) of Bloch's (1917) "Dopa"[3] reaction. These preparations were finally mounted in balsam. All scales were studied by both transmitted and reflected light and photographic records made. Appropriate control preparations were reserved for each condition. The ventral surface was carefully examined for pigmentation with a dissecting microscope before each animal was sacrificed and daily observations were made during longer experiments.

Experimental

In connection with studies previously reported (Osborn, 1940*a*), it was observed that experimentally developed melanophores appeared in random positions and patterns on the ventral surface. Pigmented spots of macroscopic size and irregular in shape, differing in intensity from gray to black, appeared here and there over any part of the ventral surface. The only position where melanophores developed with considerable regularity was at the base of the tail. In this area normal control fishes also usually possess some pigment, probably because considerable light reaches this narrow region where the surface is somewhat rounded and unprotected by fins.

The "Dopa" Reaction

This reaction first described by Bloch (1917) and later modified by Laidlaw (1932*a*) has been used for the identification of melanoblasts. Although these cells contain no melanin pigment, Bloch observed the formation of a black substance which he called "dopa-melanin" when treated with "Dopa." He believed this was due to an oxidizing ferment, dopa-oxidase, in the cell which reacted with the "Dopa." Thus, many investigators have interpreted the "Dopa-positive" cell as a potential melanophore even though it had not yet differentiated.

In order to test ventral scales for the presence of melanoblasts by the "Dopa" technique, three groups of flounders (12 fishes in each group) were chosen. The first group was black-adapted, then totally blinded and finally illuminated ventrally to insure optimum conditions

[3] Throughout this paper the term "Dopa" will be used to refer to 3–4 dihydroxyphenylalanin (levorotatory).

for rapid growth of melanophores (Osborn, 1939, 1940*a*). This treatment was continued until considerable ventral pigment had developed (Fig. 6). When a random sampling of ventral scales from such a fish was studied it became apparent that all degrees of pigmentation (melanogenesis) were represented by the various scales (Figs. 8, 9, and 10). In some scales there were no melanophores, in others the small number of melanophores had only scattered melanin granules while still others were melaninated so heavily as to be indistinguishable from scales plucked from the dorsal surface. Such scales arranged in a progressive series show all stages in the acquisition of a full complement of melanin in melanophores, suggesting that the process of pigmentation occurs in the cells *in situ* as they differentiate on a particular scale. Furthermore, there appears to be no tendency for scales adjacent to naturally pigmented areas (the edges of the fins etc.) to become pigmented first with subsequent spreading from originally pigmented surfaces. Rather, melanophores suddenly appear containing a few pigment granules quite independently of neighboring cells. In an attempt to obtain more than circumstantial evidence on this point some scales possessing no melanin-containing cells (microscopic examination—Fig. 2) and others containing but few young melanophores (the exact number and their position on the scale recorded in each instance) were subjected to the "Dopa" treatment. An average of 14 out of 20 scales from each of the 12 fishes gave a positive "Dopa" test (Fig. 3).[4] In some ventral scales positive cells were as numerous as the melanophores on dorsal scales while in other instances only scattered cells responded positively. An entirely satisfactory explanation for the failure of some scales to react positively cannot be given. Two possibilities are suggested: either these scales possessed no melanoblasts, as is apparently the case in scales occasionally found on the dorsal surface, or the technique may not be entirely dependable even though precautions were taken that the solutions were fresh and the incubation temperature accurately controlled.

The flounders in group 2 were illuminated ventrally for a shorter period (4 to 10 days), only until the first appearance (macroscopic) of partially pigmented scales here and there over the surface. For the "Dopa" test scales were chosen which possessed no melanin-containing cells or but few melanophores (again carefully recorded). In this group an average of 16 out of 20 scales per animal responded positively. The range of variation was wide as in the first group.

[4] It is of interest to note that these "Dopa" positive cells appear similar to the round melanoblasts pictured by Laidlaw (1932*b*; Fig. 5, Plate 84; and Fig. 8, Plate 85) in human skin.

The third set of flounders was not subjected to ventral illumination or any other laboratory conditions. They were used immediately without allowing time for adaptation to any unnatural background. Adequate scales were plucked from the ventral surface for each of the three types of preparations previously listed and routine "Dopa" tests were run. None of the cells of the scales used possessed microscopically detectable melanin granules. An average of 13 out of 20 scales from each fish reacted positively to "Dopa." Again the range of variation in the number of positive cells from scale to scale was wide. It is of considerable interest, however, that flounders taken directly from nature should possess numerous potential melanophores on a surface so free from melanin.

Observations Using Transmitted Light

A brief summary follows for the microscopic observations of ventral scales studied by transmitted light. Some were mounted in glycerine jelly to preserve the alcohol-soluble pigments; others were mounted in Clarite following dehydration and xylol clearing.

In glycerine mounts the numerous leucophores appear slightly opaque (Fig. 2) because of their content of guanin crystals and may be easily recognized by their relative numbers, irregular (dendritic) shape and their size. Other cells, less numerous, flattened, and smoother in contour, almost round (in fact having the same shape as those which reacted positively to "Dopa"), could be seen scattered among the leucophores. They are believed to be melanoblasts and are best seen when the iris diaphragm is partly closed. Young melanophores, containing few melanin granules, are of much the same appearance but usually are given a slightly more irregular form by the extensions of simple processes. In studying scales arranged in series progressing from those having no melanophores to scales possessing numerous melanin-containing cells there appears to be a direct correlation between the increase of melanin contained in the cell and the complexity of the processes. A coincidence observed so regularly that it should not be overlooked was that wherever several melanophores were growing in a group the absence of leucophores in that spot was strikingly obvious (Figs. 9 and 10). Viewed with reflected light this was even more easily seen. This suggests that in some way a substitution of melanophores for leucophores may take place or that conditions in the tissues favoring the generation of new melanophores may also be responsible for the degeneration of leucophores. Can it be that leucophores change into melanophores? The very existence on the dorsal surface of structures which apparently

contain both melanin and reflecting material (probably guanin) is evidence supporting the idea that two pigments may occur within a single cell (melanoleucophore—Figs. 4 and 5).

It was noted also that in scales possessing many melanophores the cells appeared to be larger and more complex with more numerous, irregular processes in contrast with other scales which had perhaps a half dozen or less melanophores usually of uniform small size and simple pattern, apparently less highly differentiated. To a certain extent the melanophores on a particular scale tend to differentiate more or less synchronously. The way in which experimental pigmentation first appears on the ventral surface of the flounder seems to be in harmony with this and with the evidence gained in the "Dopa" tests. In addition, occasional cells containing some yellow pigment (xanthophores) were seen.

Clarite mounts showed essentially the same picture except that no xanthophores were detected. The leucophores were much more transparent but could be recognized by reducing the light. The smaller round cells were also visible.

Observations with Reflected Light

Glycerine mounts viewed with reflected light showed the leucophores in clear relief but to the disadvantage of the other cells present. However, in cases where some melanophores had developed among the leucophores, the negative outline of the melanin-containing cells could be followed, aided somewhat by the absence of leucophores at that site (see previous page and Figs. 9 and 10). Now and then xanthophores were observed by reflected light.

The scales cleared and mounted in Clarite were less instructive when viewed with reflected light. Because their relative transparency reduced the clarity of the reflected image, they supplied little additional information.

Observations Concerning Regenerating Scales

In areas of injury on the ventral surface where scales had been scraped away, the newly regenerated ones appeared darkly melaninated if the fish was maintained in a physiological and experimental condition favorable to the development of ventral pigment. Such scales are black with melanophores as they appear (Fig. 7). However, if the injured flounders are white-adapted or on a pale natural background with normally alternating night and day (not excessive illumination), the regenerating ventral scales will be white with leucophores and possess no

PLATE I

Explanation of Figures

Fig. 1. White ventral surface of a freshly caught summer flounder. Note that the scales are normally covered with leucophores (containing guanin) but that melanophores fail to develop on this surface. About ⅙ natural size.

Fig. 2. Photomicrograph of a scale (mounted in glycerine) plucked from the ventral surface of a normal summer flounder. This scale possessed no melanophores. The numerous gray-appearing cells are leucophores which appear slightly opaque when photographed with transmitted light. About 20 ×.

Fig. 3. Photomicrograph of a scale which reacted positively to the "Dopa" treatment. The densely opaque cells which have deposited dopa-melanin are interpreted to be melanoblasts. Before treatment this scale appeared similar to that in Fig. 2. About 20 ×.

Fig. 4. Photomicrograph (*transmitted* light) of a small area of the tip of a scale plucked from the center of a white "excitation spot" on the dorsal surface of a black-adapted flounder. The melanophores are numerous even in this white area but are only slightly dispersed and well concealed by guanin crystals, as will be seen in Fig. 5 taken with reflected light. Cell "x" is the same structure marked for purposes of orientation in Figs. 4 and 5. About 100 ×.

Fig. 5. The same area as shown in Fig. 4. This photograph was made with *reflected* light, however, Note that the total area appears relatively white as it would on the fish in reflected light even though the scale is heavily melaninated. The reflecting guanin appears to be within the bounds of the melanophores because the cells retain a constant size and shape when viewed by reflected and transmitted light. Such a structure is referred to as a "melanoleucophore." Compare Figs. 4 and 5 cell for cell. About 100 ×.

Fig. 6. Ventral view of summer flounder blinded immediately after capture and continuously illuminated ventrally (direct light). Although this fish was illuminated only 18 days, its melanination is nearly as extensive as on the flounder shown in Fig. 7. This is due to the greater efficiency of direct illumination. One-sixth natural size.

Fig. 7. An area of ventral surface adjacent to the pectoral fin. The flounder was black-adapted, blinded, and illuminated continuously 74 days in a white tank. Widespread melanophore formation has occurred but pigmentation is blackest where regenerated scales have grown in an injured area from which the scales had been scraped. One-third natural size.

Fig. 8. Photomicrograph of the tip of a dorsal scale plucked from one of the white "excitation spots" of a black-adapted flounder. The numerous melanophores are only slightly distended during excitation despite the fact that the fish had been black-adapted several days. Scales possessing comparable melanination are commonly found in the darker pigmented areas ventrally. About 50 ×.

Fig. 9. Photomicrograph of part of a scale plucked from a lightly melaninated area of the ventral surface of a summer flounder black-adapted 7 days, blinded and placed in a white tank constantly illuminated from overhead for 12 days. The photograph was taken with *transmitted* light. The few young melanophores present appear in distinct contrast to the numerous leucophores which cover nearly the entire scale surface. Note that the leucophores are absent from the newly melaninated area. Melanophore "a" serves as a point of reference and orientation in Figs. 9 and 10. About 40 ×.

Fig. 10. Same area shown in Fig. 9. Photographed with *reflected* light, the numerous leucophores appear white as on the lower surface of the normal flounder. They contain no melanin and are true leucophores. The melanophores are visible only because they reflect the least light and appear black in contrast with the rest of the scale surface. Compare Figs. 9 and 10. About 40 ×.

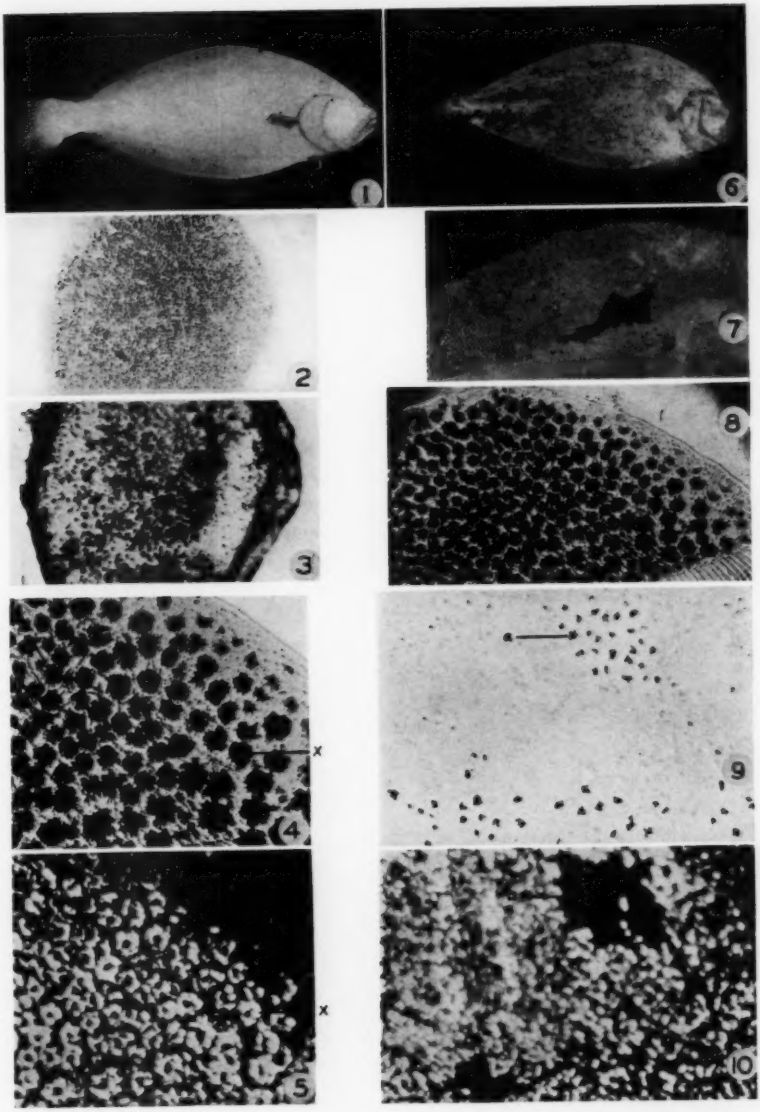

PLATE I

melanophores. On the other hand, if the dark flounders are injured, then placed immediately in the apparatus providing ventral illumination, the scales which regenerate appear as some of the darkest on the entire ventral surface. Although the reason for this is not definitely known, it may be that the numerous leucophores on the white scales tend to mask the developing melanophores during their early stages. The prompt appearance of melanin-containing cells in areas of repair far removed from normally pigmented regions lends further support to the *in situ* origin of ventral melanophores and makes the possibility of migration from previously pigmented areas seem very improbable.

Discussion

That melanophores may be grown under proper experimental conditions on surfaces naturally white and non-melaninated is now well established. The conditions favoring such growth are also known (Osborn, 1939, 1940*a*, *b*, and *c*) although many details await further investigations. The experimental requirements are two-fold: (1) That the surface in question must receive light either directly or by reflection and (2) that the physiological condition (nervous and hormonal factors) of the fish must be such that the internal environment of the normally pigmented cells favors dispersion [5] of the melanin granules (physiological darkening). Odiorne (1937) concluded that this condition also favored the slower morphological darkening in *Fundulus*. Similar findings have been reported in the lower vertebrates by Vilter (1931), Sumner and Wells (1933), Sumner and Fox (1935), Sumner and Doudoroff (1937), Sumner (1939, 1940*a* and *b*), Osborn (1939 and 1940*c*), and Dawes (1941).

The possible source of experimentally developed melanophores attracted the attention of Cunningham (1893), who saw no pigment migrating from the upper surface and so from negative evidence concluded the cells developed *in situ*.

The positive evidence presented here although partly circumstantial supports the view that these melanophores develop where they are first observed. The evidence gained from the direct observation (using both transmitted and reflected light) of small cells whose appearance and distribution agree in general with the picture seen in positive "Dopa" preparations requires no further comment. Likewise the observations concerned with the melanination of regenerated scales strongly favor

[5] That the degree of dispersion need not be maximal is shown by the fact that considerable ventral pigment may be grown on an animal whose dorsal surface is intermediate in shade. It is important to emphasize that the fish should not be in the pale phase.

the *in situ* origin of these melanophores. Furthermore, the fact that evidence gained in using the "Dopa" reaction as an indicator fits well into the other findings suggests that in this instance the reaction is significant and reliable. It is fully appreciated, however, that a positive "Dopa" reaction because of its non-specificity may in itself be of limited value or may prove to be misleading unless supported by evidence from other sources. In these experiments the "Dopa" reaction was used only to test for the presence of positive cells, thus avoiding the necessity of interpreting the implications of the chemical reaction. Because this problem is highly controversial at present some further discussion may be appropriate. That the positive "Dopa" test need not necessarily indicate the presence of a specific oxidizing enzyme ("dopa" oxidase) in the cell has been suggested by Figge (1940), who found that "Dopa" would react with other substances under test tube conditions to produce a black deposit. However, a characteristic feature of a melanoblast is that it contains some substance which will cause "Dopa" to react, forming a black material. This may be formed by a substance which oxidizes the "Dopa" or which acts as a redox substance to accelerate the auto-oxidation of "Dopa" in the absence of an enzyme. This latter possibility seems rather unlikely.

Another possibility is that the cells believed to be melanoblasts on the ventral scales may contain tyrosinase which for some reason has failed to react with tyrosine to produce pigmentation. Figge (1940) suggested that a positive "Dopa" reaction might indicate the presence in a cell of tyrosinase whose oxidizing action was inhibited by a glutathione-like substance. Such substances are known to inhibit the action of tyrosinase on tyrosine but do not inhibit the action of tyrosinase on "Dopa" (Figge, 1940). Tyrosinase actually blackens "Dopa" faster than tyrosine. It is seen then, that the observations made can be explained on a theoretical basis, although we do not, of course, know precisely what happens in the cell.

The question may be asked: "If the melanoblasts are present on the ventral surface, why do they not finish differentiation normally by manufacturing pigment?" It has been demonstrated that they do differentiate to true melanin-containing melanophores when the proper conditions are supplied. One might argue that the internal environment is the same in the ventral cells as in the dorsal cells and that the external environment differs only with regard to the amount of light which normally reaches the upper and lower surfaces of the flounder respectively. If this assumption is true, the following speculation may be offered. Is it possible that in the potential melanophores of the ventral scales a

tyrosine-tyrosinase reaction is inhibited by a glutathione-like reducing agent in the absence of light and that exposure to light (experimentally) might remove the inhibiting effect of the reducing agent and allow the enzyme to oxidize the color substrate? That the above assumption is not entirely true, however, is suggested by other observations. The internal environments for the cells of the dorsal and ventral scales are presumably alike in their hormonal constituents but not necessarily so in regard to their respective innervations. This is not known. Pouchet (1876) suggested, however, that a partial atrophy of the sympathetic system may accompany the migration of the corresponding eye during metamorphosis. In view of the present findings concerning ventral pigmentation, further experiments designed to provide new information on the possibility of the degeneration of sympathetic fibers to the ventral surface are needed.

Summary

Melanophores differentiate on the normally non-melaninated ventral surface of summer flounders when two conditions are satisfied. (1) The surface must be exposed to some light source when (2) the animal is in a physiological condition favoring darkening as witnessed by the behavior of the dorsal melanophores.

The melanophores develop "*in situ*" from potential melanophores (melanoblasts) whose presence is evidenced by the positive "Dopa" reaction, by direct observations of various stages of differentiation using direct and reflected light, by studies on regenerating scales, and by additional physiological data.

Theoretical considerations of the possible reactions involved in the experimental development of ventral melanophores and speculations as to why they are normally absent from the ventral surface are presented.

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STUDIES ON THE GROWTH OF INTEGUMENTARY PIGMENT IN THE LOWER VERTEBRATES

II. The Rôle of the Hypophysis in Melanogenesis in the Common Catfish (Ameiurus melas) [1]

CLINTON M. OSBORN

(From the Department of Anatomy, The Ohio State University)

In previous communications (Osborn, 1940*a*, *b*, and *c*) it was shown that smooth-skinned as well as scaly teleosts responded to experimental procedures by developing melanophores on their normally unpigmented surface. These findings confirm and extend the earlier works of Cunningham (1891, 1893); von Frisch (1911); Herbst and Ascher (1927) and others more completely listed in another paper (Osborn, 1941, in press).

In several experiments concerned with the experimental growth of melanophores it has been observed consistently that these cells developed most rapidly and abundantly when the fish was in a physiological condition which caused pigment granules in the normally existing melanophores to be maximally dispersed (Osborn, 1940*a* and *b* and 1941 in press). Odiorne (1937) suggested that "Any condition leading to the dispersion of pigment throughout the cell will, if maintained, promote the development of melanophores or insure their continued existence"; and further wrote that "The neurohumors which are instrumental in bringing about the pigmentary migrations in Fundulus also exert trophic influences upon the melanophores."

In the catfish it has been found that melanophores can be grown experimentally on the naturally white belly of the animal by directing light upon it while the pigmented dorsal surface is in the dark phase (Osborn, 1940*c*). If the fish is white-adapted, however, ventral illumination does not result in melanophore formation. It was previously shown (Osborn, 1938) that the melanophore-dispersing principle of the hypophysis plays a major rôle in producing the dark phase (physiological darkening) in the natural color changes of the animal. By removing the source of this secretion but maintaining constant all other conditions favoring the growth of melanophores on the white belly surface, it

[1] It is a pleasure to acknowledge that this investigation was aided in part by a grant from the Elizabeth Thompson Science Fund.

should be possible to determine whether a substance necessary in physiological darkening was also essential in the production of melanophores (morphological darkening). In the experiments to be reported here the fact that melanophores failed to grow experimentally in hypophysectomized catfishes indicates that a substance necessary to produce the dark phase is also indispensable to the experimental development of melanophores. This strongly suggests that morphological color change is not the result of physiological color change but rather that both are the product of a common underlying mechanism which effects the former change more slowly than the latter.

Materials and Methods

Common catfishes (*Ameiurus melas*) six to eight inches in length were kindly furnished me by Dr. T. H. Langlois, director of the Franz Theodore Stone Laboratory, Put-In-Bay, Ohio.[2] The laboratory stock was kept in muddy water at 12 to 18° C. in large gray tanks in an animal room where the illumination was of low intensity and darkness was maintained at night. Under such circumstances fishes have been kept over a year in excellent condition and have maintained normal pigmentation. The experimental fishes were kept in water at 10 to 12° C. during the first post-operative week and henceforth the temperature was maintained between 14 and 18° C. At this temperature they took food regularly: rolled oats daily and bits of liver or ground beef about once a week.

Illumination was directed to the ventral surface of the experimental fishes either by specially constructed glass-bottomed tubs with ceiling and sides black or white (Osborn, 1940*a*) or by reflection from white tubs brightly illuminated from above. Both of these light sources have been used successfully in growing ventral melanophores. In our apparatus illumination by reflection grows pigment less rapidly, however, because of the lower intensity of the light actually falling upon the lower surface of the fish.

The fishes, after having been lightly anesthetized in a dilute chloretone solution or stupefied by chilling, were totally blinded by enucleation and were hypophysectomized by the oral approach. Hypophysectomies were checked for completeness by reconstructions at the time of operating, by observing the post-operative color changes displayed by each fish and by examination at autopsy. When for any reason the operation was considered imperfect the data for that animal were discarded.

Some of the fishes were sacrificed at convenient intervals for microscopic study, others for chemical determinations. In almost all cases

[2] Courtesy of Mr. John Sullivan, Ohio Conservation Department.

TABLE I

	Animals alive 30 days after beginning of experiment	
	Number	Percentage of original
Group A	43	71.7
Group B	29	97.0
Group C	16	72.7
Group D	15	100.0

photographs were taken of living fishes but in certain instances additional records of preserved animals were made.

EXPERIMENTAL

In these experiments over a hundred catfishes were used representing four different physiological or operative conditions as follows:

Group A—60 fishes—totally blinded; hypophysectomized 12 hrs. later.
Group B—30 fishes—totally blinded.
Group C—22 fishes—hypophysectomized only.
Group D—15 fishes—unoperated controls.

Animals from each of the above groups were placed in each of five experimental tubs: four providing continuous direct ventral illumination (apparatus only slightly modified from that previously described, Osborn 1940*a*) and one having a white bottom which reflected light to the belly of the fishes. By having representative fishes from each of the groups in every tub, any possible effects of slight differences in temperature, light intensity, feeding, etc. were automatically ruled out. The

PLATE I

EXPLANATION OF FIGURES

Figures 1 and 2 are ventral views of two fishes described below (about ⅔ natural size). Figures 3 and 4 are lateral views of the same two fishes.

FIGS. 1 AND 3. A common catfish (*Ameiurus melas*) blinded and continuously illuminated ventrally by light reflected from the white bottom of the tub in which this experimental fish was kept for 125 days. Note dark shade and excessive ventral melanination.

FIGS. 2 AND 4. A catfish blinded, 12 hours later hypophysectomized and maintained 125 days in the white tub described above continuously illuminated. Note the pale shade and relative loss of pigment compared with blinded control Fig. 8. The fishes in Figs. 1 and 2; 3 and 4 were photographed and printed together.

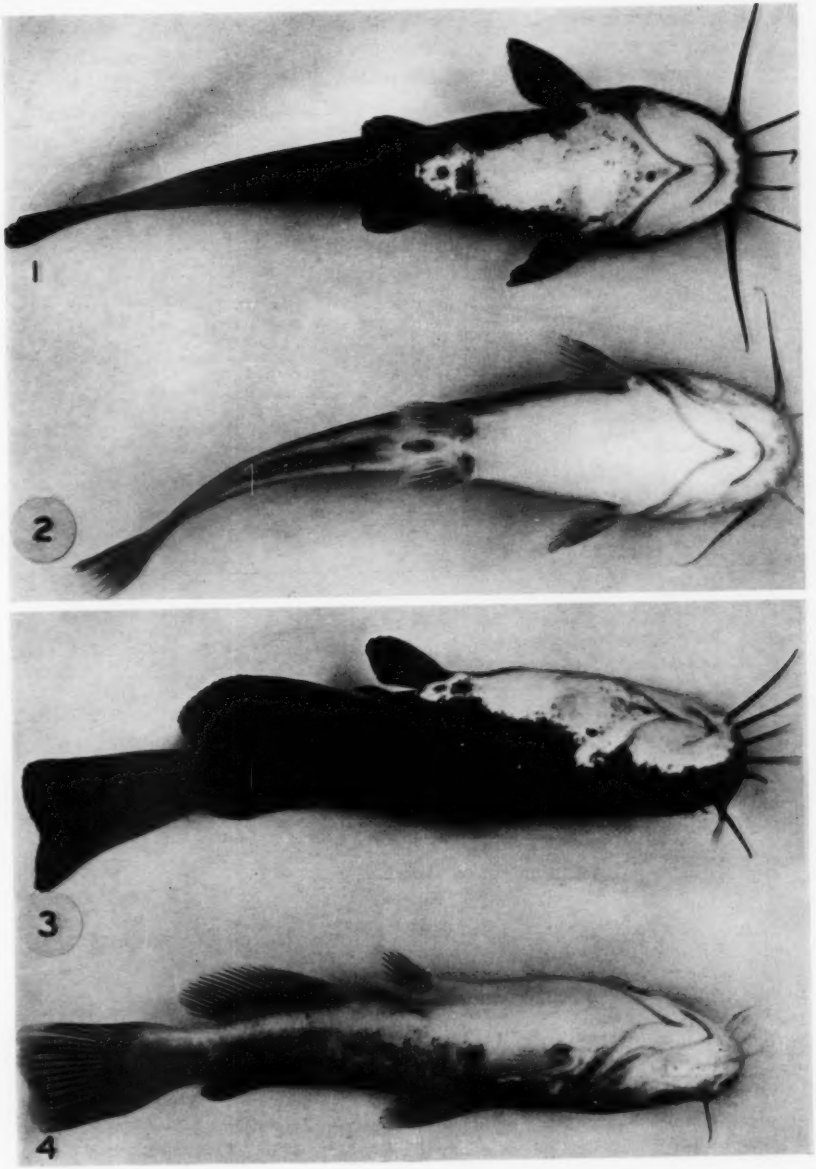


PLATE I

animals were checked twice daily for mortalities and dead or dying fishes were placed in fixative immediately and observations recorded.

Because at least a month of continuous illumination (at the intensity used) was required to develop more than a slight amount of ventral pigment in the catfish, data on fishes surviving less than 30 days were discarded. It is important, therefore, to list animals surviving the operation by 30 days. About 80 per cent of the original fishes were alive and distributed as shown in Table I.

After the first month the mortality rate decreased, presumably because the less vigorous animals succumbed earliest. After that, occasional deaths, combined with the intentional sacrifice of an animal now and then, reduced the number of experimental fishes considerably so that at the end of 180 days 51 animals (about 40 per cent) surviving in good condition were distributed in the four groups as shown in Table II.

TABLE II

	Animals alive at end of experiment—180 days	
	Number	Percentage of original
Group A	11	18.3
Group B	23	76.7
Group C	6	27.3
Group D	11	73.3

At the end of the experiment (180 days) representative animals from each group were reserved for chemical and other quantitative

PLATE II

EXPLANATION OF FIGURES

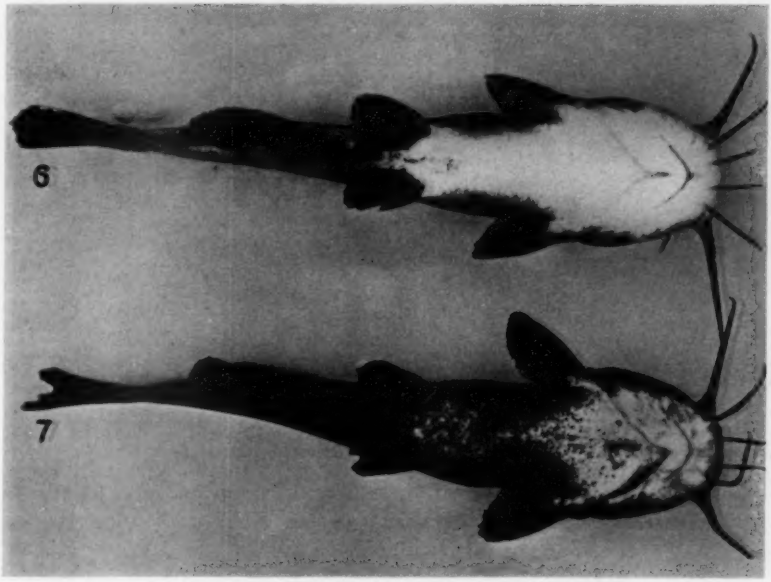
All ventral views—about ⅔ natural size.

FIG. 5. A catfish blinded and subjected to *direct* ventral illumination for 148 days. Note how the normally unpigmented white vest has become almost completely blackened with melanophores. Direct illumination grows the pigment faster than weaker reflected light. Compare with Fig. 1.

FIG. 6. A catfish (eyes intact) ventrally illuminated with direct light for 55 days. The animal remained somewhat dark-adapted to the black sides and ceiling of the tub. Note that some ventral pigment has grown, especially at the base of the anal fin. Compare with Fig. 8.

FIG. 7. A catfish blinded and ventrally illuminated (direct light) 79 days. The pigmentation is somewhat less extensive than in Fig. 5.

FIG. 8. An animal blinded and kept with the stock fishes in an unlighted tank of neutral shade 76 days. Note the dark shade resulting from blinding, but excessive pigmentation has not occurred. This fish serves as an appropriate control for some of the other animals illustrated.



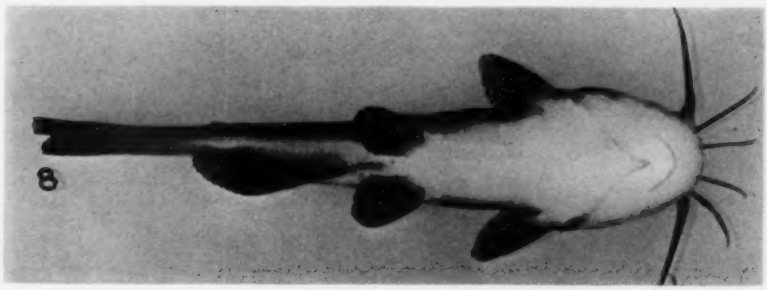


PLATE II

determinations, the results of which will be assembled in a later communication. Only qualitative results will be reported here.

Results

After prolonged treatment under the conditions described, the animals from all four experimental groups could be divided into only two categories on the basis of general macroscopic appearance.

On the one hand fishes of group B which had been blinded only (hypophysis intact) had become coal black dorsally and laterally and had developed dense ventral pigment especially around the cloacal aperture and posteriorly around the anal fin in addition to numerous spots of melanophores scattered over the normally white vest of the belly surface (Figs. 1, 3, 5 and 7). The deposition of melanin in these fishes was strikingly excessive as evidenced by the amount of black pigment which came off on one's hands when the fishes were handled for observations.[3] This never occurred in handling fishes of any of the three other experimental groups.

On the other hand, fishes of groups A (blinded and hypophysectomized), C (hypophysectomized—eyes intact) and D (unoperated) were all *pale in shade* (except those in group D in black-walled tubs) and in general not easily distinguished by original groups although in some instances those in group A seemed slightly darker than fishes in groups C and D. Whether this was a significant difference may be decided from future quantitative determinations. Of interest in our present findings is the fact that hypophysectomized animals of group A were unable to grow melanophores (Figs. 2 and 4) while those in group B, alike in all experimental details except that the hypophysis was functionally intact, grew abundant melanophores. All fishes in both groups had been totally blinded, an operation which in normal catfishes results in the pronounced darkening of the integument (Fig. 8) due to maximal dispersion of the melanin granules within the melanophores (Parker, 1934 and 1939; Abramowitz, 1936; Osborn, 1938). When animals thus blinded are hypophysectomized, however, the integument pales considerably with corresponding concentration of the melanin granules (Osborn, 1938). It appears, then, that when the pigmentary system is subjected to these two opposing influences, melanogenesis is not accelerated even though the external environment (illumination, etc.) strongly favors the growth of melanophores. Furthermore, the melanophores are not maintained normally but rather undergo gradual degeneration.

[3] This is in all probability the result of large numbers of superficial melanophores being cast off through the epidermis, a condition invariably found in catfish integuments where melanin production is going on at an accelerated rate.

Animals in groups C and D placed in white tubs remained very pale and no evidence of accelerated melanogenesis was observed although ventral illumination was continuously provided. Animals in group D were actually white-adapted normal animals (tub walls and ceiling white) while those in group C not only were white-adapted but were deprived of the hypophysis, the source of the chief melanin-dispersing factor in the normal chromatology of the catfish. Of the other fishes in groups C and D in tubs with black walls and ceiling, those in group C were very slightly darker qualitatively than corresponding fishes in white tubs while group D catfishes were rather black-adapted with a noticeable increase in pigmentation (Fig. 6).

It was noticed that the animals of groups A, C and some in D (those in white tubs) not only failed to show evidence of accelerated melanogenesis but actually appeared less heavily pigmented at the end of the experiment than stock controls.

Discussion

Two types of color change have been recognized for several years, rapid and gradual. The thesis that there is a causal relation between the phenomena of transitory and of quantitative color change referred to as "Babak's Law" recognizes as separate features the rapid color changes and those of a very gradual, less temporary type. Odiorne (1937) speaks of these as "physiological" and "morphological" color changes respectively. He found that the pigmentation of *Fundulus majalis, F. heteroclitus,* and *Ameiurus nebulosus* is "reduced through the degeneration of melanophores when these fishes are kept on white backgrounds, but tends to increase when they are kept upon black backgrounds." He also reported that "The development of pigmentation in young fishes (*Macropodus* and *Gambusia*) is retarded if they are kept on white backgrounds, but on black backgrounds the fishes become very dark." Odiorne concluded that "Morphological color changes (alterations in pigmentation) and physiological color changes (arising from pigmentary movements) are phenomena resulting from a common cause."

Other investigators (von Frisch, 1911; Vilter, 1931; Sumner and Wells, 1933; Sumner and Doudoroff, 1937; Sumner, 1939 and 1940*a* and *b*; and Dawes, 1941) have reported experiments concerning an increase or decrease in integumentary melanin. So far as the writer is aware, every case of melanin increase was associated with a condition favoring melanin dispersion in the cells, whereas decreases in melanin regularly occurred in animals maintained in the pale phase over extended

periods. These observations are in total agreement with the conclusions of Odiorne, but direct evidence to indicate that a substance active in physiological color change is also necessary for the formation of new melanophores has hitherto been lacking. The results recorded here indicate that the melanophore-dispersing substance of the pituitary gland, so important in producing the dark phase of the catfish in its normal physiology (Osborn, 1938), is also necessary for the development of new integumentary melanophores and for the maintenance of those already formed. When this substance is absent from the blood (in hypophysectomized fishes), new melanophores are not developed even when otherwise optimum conditions for their growth are maintained. This is most clearly seen in the white normally non-melaninated vest of the fish, which will become pigmented with melanophores under the conditions described in group B (Figs. 1, 3, 5 and 7), using ventral illumination. Not only did such pigmentation fail to occur in catfishes whose pituitaries had been removed, but many of the melanophores present previous to the operation underwent degeneration.

These findings suggest that the melanophore-dispersing substance circulated in the blood stream of the normal fish provides a favorable medium (internal environment) in which melanogenesis may go on. We do not yet know, of course, whether this pituitary fraction itself enters actively into the chemistry of melanin formation or whether it acts as a catalyst in some way. In this connection it is of interest to note that Fostvedt (1940) has reported that some pituitary fractions especially high in melanophore-hormone content produced marked acceleration of the oxidase system in the tyrosine-tyrosinase reaction. This was shown in hypophysectomized frogs whose legs, isolated, were incubated for specified periods of time following injection with the extract. Although this is somewhat removed from catfish chromatology it suggests, at least, how the melanophore hormone may enter into melanin formation naturally, especially in animals whose normal color change mechanism is so highly dependent upon this pituitary secretion.

Incidental to other observations, Rahn (1941) noticed in the rattlesnake that following hypophysectomy little, if any, melanin was deposited into the cells of the shedding stratum corneum. This probably indicates a failure of the melanophores to produce normal amounts of pigment in the absence of the hypophysis. Recent clinical reports by Fournier, Cervino and Conti (1941) indicate success with local injections of melanophore hormone in the treatment of vitiligo in man. Their illustrations show clearly that pigment-free patches become repigmented under administration of the hormone. This finding, together with earlier reports by With (1920), Buschke (1907) and others who

treated vitiligo successfully by stimulating the growth of pigment with light baths indicates that in the human being dual factors (*light* externally and a *hormone* internally) may coöperate in the growth and maintenance of pigment. It is interesting that similar agents are shown here to control pigment production and maintenance in a teleost.

Because facts in this field are just beginning to accumulate, anything more than speculation would be quite premature. Is it not conceivable, however, that the intermedin abundant in the mammalian hypophysis might be concerned in maintaining the degree of pigmentation peculiar to the individual and that an imbalance of this factor might be correlated with certain pathologies where active melanogenesis is characteristic?

Summary

The common catfish (*Ameiurus melas*) possesses naturally a white vest ventrally in which melanophores are only rarely found. In appropriate apparatus it is possible to grow melanophores abundantly over this naturally unpigmented area and increase the amount of pigment in other areas if the dorsal aspect (normally pigmented surfaces) of the fish is maintained in the dark phase. It is convenient, though not necessary, to continue the dark phase permanently by blinding the fish totally, a fact which "per se" indicates that the eyes are not necessary in active melanogenesis.

If the pituitary gland is removed, however, melanogenesis does not continue. In fact, melanophore degeneration sets in with the end result that the experimental fish is paler and less heavily melaninated than stock controls. This indicates that the melanophore-dispersing hormone of the pituitary gland so important in the normal color change physiology of the catfish is also indispensable to the development and maintenance of melanin in melanophores. Interpreted in another way, it suggests that morphological color change is not produced by physiological color change but rather that both are the result of a common underlying mechanism.

A possible way in which the melanophore-dispersing fraction of the pituitary may be involved in the production of melanin is discussed. It is suggested that the melanophore-dispersing hormone (intermedin) in the human hypophysis may be concerned in the production and maintenance of normal pigmentation in man.

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THE RÔLE OF ANTIFERTILIZIN IN THE FERTILIZATION OF SEA-URCHIN EGGS

ALBERT TYLER AND KATHLEEN O'MELVENY

(From the William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology)

Introduction

In recent years several investigators (Frank, 1939; Tyler, 1939*a*, 1940; Southwick, 1939; Hartmann, Schartau and Wallenfels, 1940) have obtained from sperm of sea urchins and of mollusks, a substance that reacts with the fertilizin obtained from eggs, and which is therefore termed antifertilizin. The reaction is manifested by the following effects:—(1) When it is added to a solution of fertilizin, the sperm-agglutinating property is proportionately destroyed; (2) under appropriate conditions it forms a precipitate with fertilizin; (3) it agglutinates eggs of the same or closely related species; (4) it produces a precipitation membrane on the surface of the egg jelly. These four effects are evidently due to the same substance which is obtained as a sea-water extract of moderately heated or of frozen and thawed sperm.

Several other effects of sperm extracts have been described. In the keyhole limpet and in the abalone the extracts contain a lytic agent (Tyler, 1939*a*) which has the property of dissolving the membrane normally present on the unfertilized eggs of these species and which is much more heat-labile than the antifertilizin. The evidence does not as yet enable a decision to be made as to whether it is a distinct substance or a complex that is only active in combination with antifertilizin or a higher "polymer" of antifertilizin. A somewhat similar lytic action of macerated sperm on the egg membrane was reported in amphibia (Hibbard, 1928; Wintrebert, 1933) and of a sperm extract on the cell mass and membrane surrounding the egg of the rabbit (Yamane, 1935).

An agent that inhibited the activity of the spermatozoa was obtained by Southwick (1939) in the supernatant from centrifuging concentrated but not dilute sperm suspensions of the sea urchin. Identity of this agent with antifertilizin has not been established nor has the possibility been excluded that the effect is due to some simple agent such as increased acidity, CO_2, etc. A similar activity-inhibiting action has been

reported in sea urchins by Hartmann, Schartau and Wallenfels (1940) for a methyl alcohol extract of sperm that does not contain antifertilizin (the agglutinin-neutralizing agent). They also find that the extract neutralizes the stimulating effect of egg water on sperm activity and the similar action of echinochrome which they had earlier reported to be the sperm-activating agent in egg water. Since their findings with echinochrome have not been duplicated in other species (Tyler, 1939*b*; Cornman, 1941), and since they have not as yet disposed of the possibility suggested by Cornman that rise in pH might be responsible for their results, it would be desirable to have further evidence before the effect of their methanol sperm extract may be accepted without reserve.

Another effect reported by Hartmann, Schartau and Wallenfels (1940) in the sea urchin is the dissolving of the jelly coat of the egg by the action of sperm extract. They find in *Arbacia pustulosa* that addition of concentrated sperm extract or of live sperm causes the disappearance of the egg jelly and we have been able to confirm this in *Strongylocentrotus purpuratus*. But, according to our observations, this disappearance does not appear to be due to solution of the jelly. When sperm extract is added to a suspension of eggs there is formed on the surface of the jelly a precipitation membrane which, in concentrated extract, gradually increases in thickness and contracts until it reaches the surface of the egg. This precipitation membrane is evidently formed by interaction of the antifertilizin in the sperm extract with the jelly. The disappearance of the latter in concentrated extracts is most simply attributable to its incorporation in the precipitation membrane and to the considerably smaller volume it occupies in precipitated rather than in gel form. As the precipitation membrane contracts the egg may, particularly when disturbed, break out of it. When undisturbed it may contract to the surface of the egg from which it is then not readily distinguishable. The disappearance of the jelly under the influence of concentrated suspensions of live sperm is likewise attributable to combination with the antifertilizin on the sperm. There does not, then, appear to be, as yet, any necessity for the assumption of a jelly-dissolving agent in the sperm extract.

In the present work the term antifertilizin is applied to that substance derived from sperm that produces the effects listed in the first paragraph. A similar antifertilizin has been obtained from eggs (Tyler, 1940), but it will not enter into the present account. The principal question at issue here is whether or not the antifertilizin of sperm is concerned in the fertilization reaction. Several facts strongly favor the presumption that it is intimately involved. In the first place it is tissue

specific, being obtainable from no other tissues (Frank, 1939). It is, however, not very highly species-specific, since cross-reactions are obtained between species that do not cross-fertilize (Hartmann, et al., 1940). This would mean that it is not primarily responsible for the species-specificity of fertilization, but this does not exclude the possibility that it is an integral part of the fertilization process. Another fact favoring its involvement is that it is evidently present on the surface of the spermatozoön. Since, in solution, it reacts with fertilizin, it most likely is the substance on the spermatozoön that reacts in the agglutination of the sperm and therefore must form at least a part of the surface. Furthermore, fertilizin has been shown (Tyler, 1941) to serve as an aid to fertilization and may possibly be an essential agent in the process. Antifertilizin, since it reacts with it, would then be expected to have a complementary rôle.

For a direct test of the significance of antifertilizin, it would be desirable to remove it completely or partially from the sperm by some non-injurious method and to examine the fertilizing capacity of the treated sperm. We have been able, in the experiments reported here, to remove antifertilizin partially without appreciable damage to the sperm. This, as the results show, causes a considerable impairment in the fertilizing capacity of the sperm.

Materials and Methods

Two species of sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus anamesus*, were employed in these experiments. Sperm and egg suspensions were prepared by removing the gonads to sea water and straining the shed sex cells through bolting cloth of appropriate mesh. The concentration of the sperm suspension was usually determined from the increase in volume after removal of the remains of the testes and is expressed as the percentage content of "dry" sperm.

The antifertilizin concentrations in the extracts were determined roughly by the intensity of the egg agglutination reaction and more accurately by the amount required to neutralize one unit (as defined by Tyler and Fox, 1940) of fertilizin (sperm agglutinin). In all the tests the pH of the solutions was checked and adjusted where necessary by means of the glass electrode.

The respiratory rate of the sperm was employed as an index of the extent of damage produced by the various treatments. The measurements were made in the Barcroft-Warburg apparatus with the cylindrical type of vessel previously described (Tyler and Humason, 1937). To

avoid possible effects of CO_2 and variation in pH, glycylglycine (Tyler and Horowitz, 1937) was used as a buffer in carbonate-free sea water.

Removal of Antifertilizin

We found that antifertilizin could be removed from the sperm by slight acidification of the suspension and also by mild warming. The antifertilizin is obtained in the supernatant after centrifugation of an acidified sperm suspension but not in that of the control. When highly concentrated control sperm suspensions are centrifuged, particularly after aging, some antifertilizin may be obtained in the supernatant, as Southwick (1939) reported. This may mean that antifertilizin normally goes slowly into solution or that centrifugation of the concentrated suspensions involves some damage and consequent liberation of antifertilizin.

Antifertilizin is obtained from sperm suspensions acidified to pH 6 or lower. The more acid suspensions yield the more concentrated solutions. One experiment with *Strongylocentrotus* sperm may be cited. Samples of a 10 per cent suspension were acidified to pH 6, 5.6, 5.1, 4.5 and 3.5. After one hour the suspensions were brought back to the control pH (7.9) and centrifuged. The control supernatant was clear while those from the acidified suspensions were increasingly opalescent. Tested on eggs the control showed no reaction while the supernatants from the acidified samples gave precipitation membranes and agglutination which increased with increase in the degree of acidity to which the samples had been exposed. Tests of their ability to neutralize fertilizin gave the following approximate titres for the antifertilizin concentration in the supernatants of the acidified samples: ¼, ½, 1, 4 and 32 units respectively. The spermatozoa were all immotile in the sample that had been exposed to pH 3.5 and partly so in the pH 4.5 sample, while those exposed to the higher pH's showed considerable activity.

These results restricted then the investigation of the treatment required for the impairment of fertilizing capacity to the range between pH 5 and pH 6. A number of tests were run at various pH's within this range and with various times of exposure. All of these showed a considerable reduction in the fertilizing capacity of the treated sperm. Similar results were obtained by heating the sperm at 30° to 33° C. for 5 to 10 minutes. The data need not be presented here since only that part which was obtained along with the respiration measurements is of particular significance. In practically all of these tests the treated sperm were found to be quite active, although in general not as active as the controls. However, differences in activity of spermatozoa are hard to

estimate by direct observation. A more objective and quantitative method consists in measurement of the respiratory rate.

Fertilizing Capacity and Respiratory Rate of Antifertilizin-Poor Sperm

Determinations were made, therefore, of the rate of oxygen uptake of the treated and control sperm along with tests of their respective fertilizing capacities. The results of five experiments are presented in Table I. Heat treatment was employed in one of these and acidification in the rest. The measurements were made in duplicate in each experiment, and both treated and control sperm were samples of the same original suspension. The control oxygen consumption values vary rather considerably in the different experiments. This variation is probably due to a number of factors such as error in initial determination of sperm concentration, variation in original condition of sperm, in its aging, etc. For the present purposes, however, this variation is of no particular significance, since comparison of treated and control sperm is made in each experiment. The duplicate runs in each experiment are in close agreement, which is to be expected since sperm suspensions can be quite accurately sampled and since the spermatozoa respire at a sufficiently high rate to make the instrumental errors relatively small.

In none of the experiments listed in Table I was the respiration of the treated sperm equal to that of the control. The highest values were 80 per cent of the control in experiments 1 and 5 and the lowest value was 25 per cent of the control in experiment 4. The treatment is, then, not entirely non-injurious to the sperm. However, a considerably greater impairment of fertilizing power results from the treatment. The fertilizing capacity of the treated sperm is listed in the last column of the table in terms of the amount required to give the same percentage fertilization, between 1 and 99 per cent, as is given by one part of the control sperm. These values are obtained from the results of inseminating samples of the same batch of eggs with serial dilutions of the control and treated sperm taken from the manometer vessels. The two figures for each experiment cover the range of variation. Thus, in the first experiment, the amount of treated sperm required to give the same percentage fertilization as the control is four to eight times the amount of the control sperm. For comparison, the next to the last column of the table gives the calculated amount of treated sperm that would have the same respiratory rate as one part of control sperm. This value is, in each experiment, considerably less than the value for the amount of sperm having a fertilizing capacity equal to one part of control sperm.

TABLE I

Respiratory rate and fertilizing capacity of sea-urchin sperm after antifertilizin-liberating treatments.

Experiment	Treatment	O_2 consumption ($mm.^3/hr./cc.$ of 1 per cent sperm)		Amount of treated sperm equivalent in resp. rate to 1 part of control sperm	Amount of treated sperm equivalent in fertilizing capacity to 1 part of control sperm
		Treated sperm	Control sperm		
1. *Strongylocentrotus*	1½ hrs., at pH 5.3	14.3, 13.2	16.5, 17.9	1.25 parts	4 to 8 parts
2. "	1 hr. at pH 5.1	2.46, 2.58	9.17, 9.47	3.7 parts	64 to 128 parts
3. "	1 hr. at pH 5.5	8.12, 7.36	11.51, 10.72	1.44 parts	16 to 32 parts
4. "	10 min. at 32° C.	3.10, 3.35	13.19, 13.24	4.0 parts	>128 parts
5. *Lytechinus*	1 hr. at pH 5.4	0.54, 0.58	0.74, 0.67	1.26 parts	16 to 32 parts

In other words, there is, as a result of the treatment in each case, a very much greater reduction in the fertilizing power than in the respiratory rate.

It is evident, too, from the data that a considerable impairment of fertilizing power would be obtained following a treatment that resulted in no reduction in respiratory rate. That we have not, as yet, succeeded in finding the proper treatment which would give that result is not surprising in view of the variability of the sperm in the different experiments and the fact that the difference is rather small between treatments giving no effect and those giving a definite reduction in fertilizing capacity. The present results, however, suffice to show that an impairment of fertilizing power can be obtained that is disproportionately great when compared with the respiration of the sperm. This impairment cannot, then, be accounted for by a decrease in activity of the spermatozoa. It might possibly be interpreted in a rather complicated manner by the supposition that a corresponding fraction of the sperm are rendered non-respiring and non-fertilizing while the remainder have an increased respiratory rate. This would mean that the effect on the individual spermatozoa would be all or none and that mild treatment would have a stimulating effect on the respiration of the suspension. There is no evidence for this. The most reasonable interpretation is that the impairment of fertilizing capacity is correlated with the loss of antifertilizin which was shown to result from the treatment.

In the experiments described here antifertilizin is present in solution in the treated sperm suspension. To determine whether its presence might affect the results, antifertilizin was added to untreated sperm in the same or slightly greater amounts. This was found to have no effect on the fertilizing capacity of the sperm. On the other hand, when concentrated antifertilizin solutions are employed an inhibition of fertilization can be obtained, as Frank (1939) and Hartmann, Schartau and Wallenfels (1940) have shown. This inhibition occurs more readily when the eggs are first treated and is evidently due to the presence of the precipitation membrane that forms on the surface of the jelly. When this membrane is incomplete or torn the egg can be fertilized, as was previously reported in the case of treatment with the antifertilizin obtained from eggs (Tyler, 1940).

Antigenicity of Antifertilizin and Action of Antisera

In order to obtain further information on the location of antifertilizin and on its rôle in fertilization, attempts were made to produce antibodies to it. Preliminary immunization experiments showed that high

titer agglutinins could be obtained in rabbits by the injection of whole sperm of the sea-urchin. Immunization with antifertilizin solutions likewise was found to induce the formation of specific agglutinins for the whole sperm as well as precipitins for the antigen in solution.

Antisera were produced against *Strongylocentrotus* and *Lytechinus* antifertilizin. The procedure and results in one experiment with *Lytechinus* follow. A solution of antifertilizin was prepared by extraction of a 25 per cent sperm suspension at pH 4.5 for two hours. The content of organic solid was determined on a sample that had been dialyzed against distilled water and was found to be between 15 and 20 mg. per cent. The rabbit was given seven intravenous injections totaling 23 cc. within a period of two weeks and was bled two weeks after the last injection. The antiserum showed by the ring test a precipitin titer of 8. Tested on a one per sperm suspension it showed an agglutinin titer of 512.

The production of agglutinins by injection of antifertilizin means not only that the substance is antigenic but is probably a surface antigen of the sperm. An examination of the agglutinates shows that the spermatozoa are stuck by their tails as well as by their heads. The antifertilizin, therefore, does not appear to be restricted to a particular location on the surface of the spermatozoön. It should also be noted here that extraction at pH 4.5 removes only a small part of the antifertilizin from the sperm since subsequent freezing and thawing or brief heating of the residue yields at least ten times the amount obtained in the acid extract. Also the acid-treated sperm are still agglutinable by antisera and by egg water.

The antigenicity of antifertilizin supports the view that it is a protein. Other evidence (to be presented in detail later) consists in its non-dialyzability, precipitation with $(NH_4)_2SO_4$, inactivation by heat and acidity, and the fact that it gives the common (xanthoproteic, Millon's and biuret) color tests.

The effect of the antiserum on fertilization was examined by insemination of eggs in its presence. Controls were run with normal rabbit serum. The sera were adjusted to sea-water salinity by the addition of an equal volume of concentrated ($1.73\times$) sea water, and equal volumes of egg and sperm suspensions were added. In all cases where the sperm was diluted to the minimum for 100 per cent fertilization in the controls, no fertilization was obtained in the antiserum. With the dilutions of sperm employed, agglutination is greatly retarded and may even fail to occur in the antiserum. The spermatozoa have not then, to any great extent, been rendered inaccessible to the eggs by incorporation in

agglutinates. The inhibition of fertilization may therefore be considered to be due to the neutralization of antifertilizin on the sperm by its antibody in the antiserum.

Discussion

The results presented here show that antifertilizin is involved in the fertilization process. In order to decide whether or not it has an indispensable rôle, one would like to have some more direct evidence such as the complete and reversible removal of antifertilizin might supply. But complete extraction without destruction of the sperm has not as yet been accomplished. From the present evidence it is reasonable to regard antifertilizin as involved in an initial step that facilitates fertilization but which may or may not be an essential part of the process. This initial step is evidently the reaction with fertilizin. In a previous article (Tyler, 1941), it has been shown that the presence of fertilizin on the egg serves as an aid to fertilization. Antifertilizin may, then, be considered to have a similar rôle in the case of the spermatozoön. For this purpose it is not effective when present in solution but only on the spermatozoön. Partial removal of the antifertilizin or its neutralization by means of an antiserum or by means of fertilizin results in a decrease or even complete suppression of the fertilizing power of the sperm. As an interpretation for the fertilization-facilitating action of fertilizin (Tyler, 1941) it was suggested that, in the form of a gel around the egg, it has a few superficial combining groups available which serve as the initial trap for the sperm but which do not neutralize all of the reacting groups (antifertilizin) on the sperm before the latter has reached the surface of the egg. On this basis the decrease in fertilizing power of the treated sperm may be interpreted to mean that, with fewer reacting groups available, there is more likelihood that they will all be neutralized before the spermatozoa reach the egg surface.

Summary

1. Acidification of sea-urchin sperm suspensions to below pH 6 or brief heating above 30° C. liberates into the solution the substance termed antifertilizin which is defined by four manifestations of its reaction with fertilizin; (a) neutralization and (b) precipitation of the latter, (c) agglutination of eggs, (d) formation of precipitation membrane on egg jelly.

2. The treatment results in a marked decrease in the fertilizing power even when the time and intensity of exposure are not sufficient to immobilize the sperm.

3. The rate of oxygen consumption of sperm, that had been exposed to mild acid- or heat-treatment, was found to be very little affected in comparison with the effect on the fertilizing power. Short extrapolation permits the conclusion to be drawn that a considerable reduction in fertilizing capacity can be obtained with no reduction of activity of the spermatozoa.

4. Injection of antifertilizin solutions into rabbits results in the production of an agglutinin for the intact sperm. This shows that the substance is a complete antigen and supports the views that it is a protein and a component of the surface of the spermatozoön.

5. Fertilization is inhibited by antisera to antifertilizin.

6. Antifertilizin is considered to be concerned in an initial (perhaps essential) step in the union of the gametes whereby the spermatozoön is entrapped by the complementary, specific reacting substance, fertilizin, on the egg; and the above inhibition experiments are interpreted on the basis of a decrease in the number of reacting groups available on the spermatozoön.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XVIII. Esterases in the Grasshopper Egg[1]

LOREN D. CARLSON

(From the Zoölogical Laboratories, State University of Iowa)

The respiratory quotients obtained for the grasshopper egg during all except the first few days of its development are characteristically those of an organism metabolizing fat (Bodine, 1929; Boell, 1935). Slifer (1930) has shown that the amount of fat in the egg measured in terms of fatty acid after saponification decreases during the phases of active development. The determination of the amounts of "lipoidal" substance that form the centripetal layer when a saline extract of these eggs is centrifuged also demonstrates a decrease in volume during embryonic growth (Bodine et al., 1939). Further, the potency of this "lipoidal" layer to activate the proenzyme, protyrosinase, varies in a different fashion than its change in volume (Bodine et al., 1939). These facts add an interest to the study of the types and activities of lipolytic enzymes present in the egg of the grasshopper (*Melanoplus differentialis*) during its embryogeny.

Materials and Methods

Grasshopper eggs were collected daily and kept at 25° C. either in the pods or separated upon damp sand within covered glass dishes. Under these conditions the eggs go into diapause within a month (Slifer, 1931). This block in development was interrupted by keeping the eggs at 5° C. for three months and then transferring them to 25° C., at which temperature they hatched in 18 days. The eggs for experiments were washed, sorted, and sterilized with 70 per cent alcohol for ten minutes (eggs 0 and 5 days of age were not treated with alcohol), rinsed and ground in a glass mortar. The ground eggs were made up to designated volume in a glycine-NaOH buffer mixture. This egg brei was centrifuged and the lipoidal or centripetal layer removed, as were the shell fragments (*A* and *C* layer of Bodine and Allen, Fig. 1, 1938). The remainder was made up to volume with the buffer mixture. Removal of the *A* and *C* layers did not alter the enzyme activity.

[1] Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

Amounts of the extract were added to a 50 cc. Erlenmeyer flask containing substrate and allowed to stand at 25° C. unless otherwise noted. Varying concentrations of enzyme and substrate were used, the total volume of the reaction mixture being 6 cc. After a period of time, the reaction was stopped with 25 cc. of a 2 per cent phenol solution and the mixture titrated with 0.05 N HCl until methyl red, used as an indicator, turned pink. Although the H ion concentration changed during the experiments, the addition of the phenol in buffer brought the pH back to the alkaline side and the HCl titre then was a measure of the NaOH neutralized during the reaction and was equivalent to the acid formed. The amount of acid thus formed is considered a measure of the rate of hydrolysis and an index of the amount of enzyme present. The equivalents of acid produced are not strictly rate values in the case of tributyrinase (Bodansky, 1937). Because of the difficulty in determining how much of the substrate was properly emulsified, a more accurate measure of rate was not practicable. Controls were duplicates of the experimentals with the exception that the enzyme extract was heated at 100° C. for 5 minutes. Reaction mixtures containing no substrate or enzyme were also tested and gave values equal to those of the control. Shaking the flasks during the reaction period did not change the rate of the hydrolysis.

The buffer mixture contained 0.1 N glycine and 0.1 N NaOH in the ratio of 9 to 1, 15 per cent glycerol, and enough NaCl to make the solution 0.17 M with respect to NaCl. The addition of salt was necessary to prevent precipitation of proteins in the extract. The phenol was dissolved in the glycine-NaOH buffer and was never used after it had acquired a brownish tinge. The methyl butyrate (Eastman Kodak), 2 per cent by weight, and the tributyrin (Eastman Kodak) and olive oil, 4 per cent by weight, were made up in the buffer containing glycerol and NaCl. Previous workers have experienced difficulty in making up tributyrin solutions which gave consistent results. Seventy milligrams of a commercial dispersing agent (Daxad No. 11) [2] per 100 cc. of solution will stabilize a tributyrin emulsion.

The method was checked using known amounts of butyric acid in place of the lipid in the protocol and the probable errors of the means of ten determinations at four concentrations between 0 and 2×10^{-4} M butyric acid were less than 2 per cent of the mean in every instance. The amount of hydrolysis of an excess of methyl butyrate (3 cc. of 2 per cent) increases linearly with extracts of one to thirty diapause eggs. The amount of acid produced by one cc. of an extract (20 eggs per cc.) was linear with time for 4 hours. When tributyrin was used as a sub-

[2] Furnished by Dewey and Almy Chemical Co.

strate, an extract of 2 eggs (diapause) would produce as much acid in 2 hours as an extract of 30 eggs would produce from methyl butyrate. The amount of acid produced from 3 cc. of 4 per cent tributyrin was proportional to the concentration between 1 to 5 eggs per cc. The reaction on tributyrin was linear with time only for the first hour. In making the following determinations, 1 cc. of an extract containing 20 eggs per cc. with 3 cc. of 2 per cent methyl butyrate in a reaction period of 2 hours and 1 cc. of an extract containing 2 eggs per cc. with 3 cc. of 4 per cent tributyrin in a reaction period of one hour at 25° C. were used as test reactions.

In a number of experiments direct titrations of reaction mixtures were made with 0.05 N NaOH to determine the extent of hydrolysis. In these the pH was first adjusted by the addition of acid or alkali and titrations made to maintain this H-ion concentration. A Leeds and Northrop pH meter with a glass electrode was used in these titrations. The time course of the reactions under these conditions at steady pH values between 4.5 and 8.0 was similar to that when the method described above was used.

Experimental

Enzymes are present in the grasshopper egg which will catalyze the hydrolysis of methyl butyrate and tributyrin but not olive oil. The enzymes are designated as methyl butyrinase and tributyrinase respectively in the following discussion although other substrates may be attacked by these enzymes. According to the nomenclature of Oppenheimer (1936), both are esterases; the one more specifically a lipase since it splits a glycerol ester of the fatty acid. The amounts of the two lipolytic enzymes vary independently during the development of the grasshopper egg (Fig. 1).[3] The methyl butyrinase activity is high at the start of development and then drops markedly between the tenth and fifteenth day. A steady level is then maintained during the diapause or inactive stage. Upon resumption of development a slow decline in activity takes place. Tributyrinase action, however, remains at the same level during prediapause and diapause and then drops rapidly in postdiapause development. An extract from a single grinding was used on both substrates in each of the ten determinations represented by the averages in the figure.

Two types of experiments were used to determine the relative amounts of the lipolytic enzymes being studied in the embryo and yolk

[3] In preliminary work (Carlson, 1940) the enzyme extracts used were so concentrated that the changes in activity were obscured.

constituents of the egg. Early prediapause (6-day) eggs were irradiated at 1000 roentgens, which is known to prevent the embryo from developing but to have no visible effect on other constituents of the egg (Evans, 1936).[4] The oxygen consumption of eggs treated in this manner decreases until the time of diapause. The O_2 uptake is low during diapause, and when the diapause is broken the oxygen uptake increases for the first two days and then remains constant (Bodine,

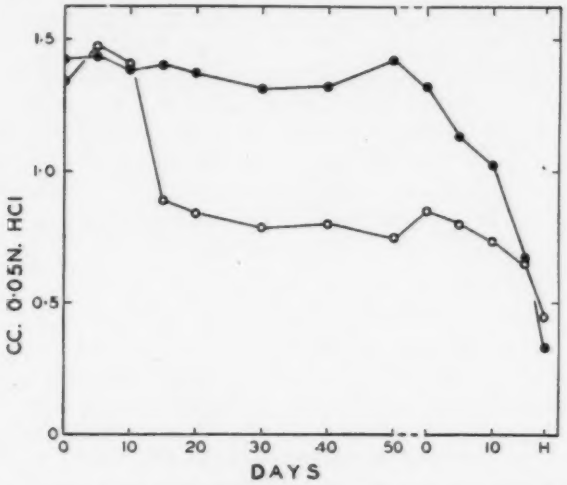

FIG. 1. Average esterase activity of five lots of eggs at each of the developmental ages shown. Ordinate, the equivalents of acid produced by hydrolysis of the esters in cc. of 0.05 N HCl; abscissa, time in days at 25° C. since laying followed by the time in days at 25° C. after termination of the diapause by exposure to 5° C. for three months. Open circles, the activity of an extract of 20 eggs in two hours with methyl butyrate; closed circles, that of an extract of two eggs in one hour with tributyrin as a substrate.

Carlson, and Ray, 1940). No significant difference could be shown between the enzyme content of the irradiated eggs and that of the controls. Determinations were carried out for 30 days after the irradiation.

In postdiapause, the embryos could be dissected from the egg and determinations made of the lipase content of the embryo and other egg constituents. The dissections were carried out in the buffer mixture. The embryos were freed from as much adhering yolk as possible and transferred with a minimum of fluid to a mortar and ground with a

[4] Dr. T. C. Evans irradiated the eggs for the author.

small amount of sand. The remaining yolk and shells were also ground and used with the dissection fluid in the determinations. There was some difficulty in freeing the embryos of yolk, but in all cases this was done as completely as possible. The amounts of enzyme in embryo plus yolk, etc. were always less than those of the whole eggs. The lipolytic enzymes seem to be associated with the yolk or its derivatives until just previous to hatching (Table I). The yolk removed in later stages of development usually included parts of the gut that could not be adequately separated. The presence of some esterase in the 5-day post-diapause embryos is attributed to the fact that the yolk and embryos were especially hard to separate at this stage.

Although the time course of reactions was similar at different pH values, the extent of hydrolysis of tributyrin was markedly affected.

TABLE I

Days Post-diapause	Embryo	Yolk etc.	Substrate
	Per cent	*Per cent*	
0	0	100	Tributyrin
5	19.3	80.7	
10	0	100	
15	0	100	
18	100	0	
0	0	100	Methyl butyrate
5	20.3	79.7	
10	8.7	91.3	
15	0	100	
18	100	0	

Reactions were carried out at pH 4.5, 5.0, 6.0, 7.0, and 7.5 by titrating frequently with 0.05 N NaOH. In Fig. 2, curve *A* shows the total amount of alkali used in this procedure over a one-hour period with the enzyme from two eggs reacting with 3 cc. of 4 per cent tributyrin. Similarly, curve *B* shows the result of experiments using the extract of 20 eggs with 3 cc. of 2 per cent methyl butyrate. The pH for maximum tributyrinase activity is at 6 while the H-ion concentration affects the methyl butyrinase activity to a lesser degree. This dissimilarity in the effect of pH on the activity of the enzymes studied is one of several differences noted. No explanation of the difference in the values for the rate of methyl butyrate hydrolysis when the pH is kept constant and when it is allowed to change is at hand.

The effect of heat treatment on the enzyme extract as well as its effect on the amount of hydrolysis was determined. In the former case

the lipolytic activity of the extract was affected differently for the two substrates. The activities of extracts were tested at 25° C. after ten-minute exposures to temperatures between 25 and 85° C. The ability to split methyl butyrate was diminished by temperatures higher than 55° C. while tributyrinase activity was unchanged after exposures to 65° C. (Fig. 3).

When the reaction mixtures were kept at temperatures varying from 0° to 45° C., the amount of hydrolysis of the two substrates differed in

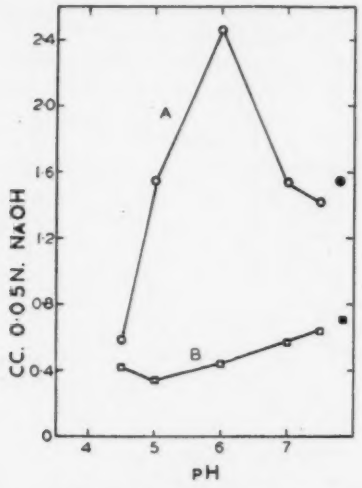

FIG. 2. The amount of hydrolysis at various H-ion concentrations. The reactions were kept at the pH noted by continuous titrations with 0.05 N NaOH. ○, the hydrolysis of tributyrin by an extract from two eggs over a period of one hour; □, methyl butyrate split by an extract of 20 eggs at the end of 2 hours. Solid symbols represent amount of acid formed when the mixture was allowed to react over the total time. Four to seven experiments averaged in each point. Reactions at room temperature.

a striking manner. The hydrolysis of methyl butyrate increased with temperatures up to 45° C. (Fig. 4*A*) and between 0° and 35° a μ value of 5700 calories was obtained (Fig. 4*B*). The Q_{10} over the corresponding range averaged 1.4. The tributyrinase activity showed a maximum at 25° C. with a decrease on either side of this temperature (Fig. 4*A*). The Q_{10} value between 5 and 15° C. is 1.97; between 15 and 25°, 1.47, using the amount of acid produced per unit time as a rate value. The values shown in the figure were obtained using an extract made in the

following manner: the eggs were ground and diluted to a volume so that the concentration was 40 eggs per cc. When this was allowed to stand a considerable precipitate was formed. This was centrifuged off and the supernatant fluid diluted to a volume corresponding to 20 eggs per cc. This resulting extract still retained its tributyrinase activity, but the methyl butyrinase reaction was reduced to one-sixth to one-fifth of that of an extract prepared in the usual manner. Falk and Sugiura (1915) were able to separate esterase and lipase materials in the castor bean, the one soluble in distilled water, the other in NaCl solution. The temperature relationship to activity of the enzyme is similar to that reported by Fiessinger and Gajdos (1936) working with an extract of the larva of *Galleria mellonella*. Their extract showed maximum activ-

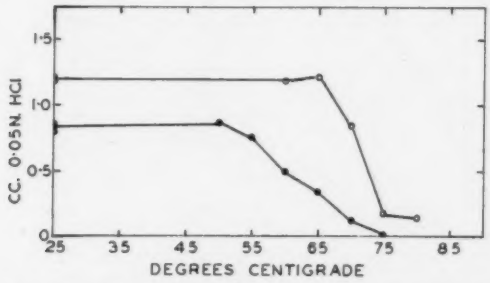

FIG. 3. The effect of temperature on the activity of the enzyme extract. Ordinate, equivalents of acid produced in cc. of 0.05 N HCl in one hour for tributyrinase and two hours for methyl butyrinase at 25° C.; abscissa, temperature in °C. to which the extract was exposed for 10 minutes. Closed circles, the hydrolysis of methyl butyrate; open circles, the hydrolysis of tributyrin. Methyl butyrinase from 20 eggs; tributyrinase from 2 eggs.

ity between 18° and 25° C. and declined at temperatures above or below this range.

Most observations concerning esterase activity indicate that the calcium ion, sodium oleate and albumin accelerate the activity of the enzymes. This is not found to be the case in extracts of the grasshopper egg. Sodium oleate reduces the lipolytic activity of the preparations used in these experiments. Calcium chloride has no effect on the enzyme but counteracts in part the effect of sodium oleate (Table II). Neither of these substances has any effect in stabilizing the pH. Albumin was not used since the extract was rich in protein. Attempts to show hydrolysis of olive oil with addition of sodium oleate and the calcium ion to the egg extracts at 25° C. and 35° C. were without success.

Various esterases are affected differently by such compounds as

phenol, quinine, atoxyl and sodium fluoride (Falk, 1924; Oppenheimer, 1936). Curiously, extracts from pancreas, liver and kidney are inhibited in their action on tributyrin in a diverse manner by quinine and atoxyl (Falk, 1924). The effect of 0.5 per cent phenol, NaF and quinine were tested on the esterases obtained from the grasshopper egg.

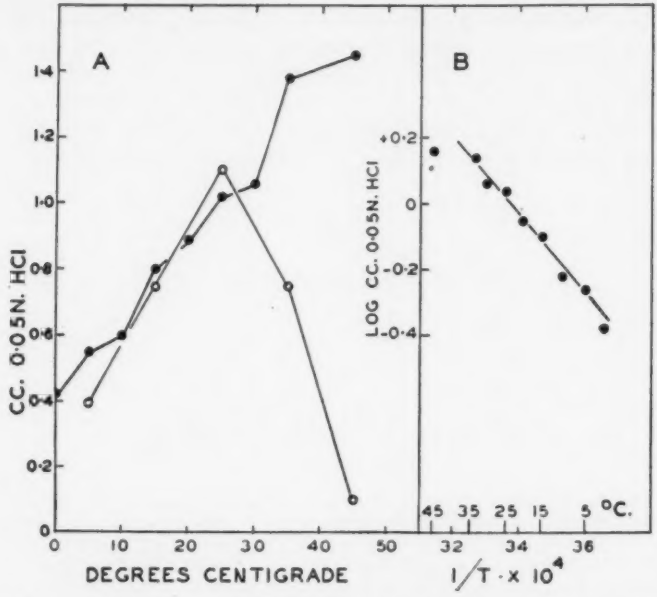

FIG. 4. The amount of hydrolysis of methyl butyrate and tributyrin at different temperatures. In *A*, the ordinate gives the equivalents in cc. of 0.05 N HCl; the abscissa, the temperature at which the reaction took place. ○, tributyrinase reaction using an extract of 2 eggs with 4 per cent tributyrin for 1 hour; ●, methyl butyrinase from 20 eggs reacting with 3 cc. of 2 per cent methyl butyrate for 2 hours. *B* shows the data for methyl butyrinase plotted as log concentration of HCl, ordinate, versus the reciprocal of the absolute temperature × 10^4, abscissa. The points are average values of ten determinations at each temperature. The straight line in *B* is fitted by the method of least squares. The μ value between 0 and 35° C. is approximately 5700 calories. For further description see text.

The results are summarized in Table III. Both methyl butyrinase and tributyrinase are inhibited by quinine and NaF. Only tributyrinase is inhibited by the 0.5 per cent phenol; methyl butyrinase activity is stimulated. Two per cent phenol will completely block both reactions. Fiessinger and Gajdos (1936), in studies on the esterase obtained from the larva of *Galleria mellonella*, found their enzyme extract unaffected by

phenol and quinine and strongly inhibited by NaF in the same concentrations as noted above with tributyrin as a substrate.

Discussion

The expectation that the grasshopper egg contains an enzyme capable of hydrolyzing triglycerides of higher fatty acids was perhaps based on a fortuitous assumption. The presence of such an enzyme in an animal metabolizing fat as the R.Q. indicates (Bodine, 1929; Boell, 1935) and consuming 60.3 per cent of its initial store of fats during development (Slifer, 1930) seemed highly probable. No evidence for this enzyme could be elicited using the methods described. The activity

Table II

	0.05N HCl (in cc.)			
Control	Control + NaOl 0.2 cc. −0.4%	Control + NaOl + $CaCl_2$ 0.4 cc. −2%	Control + $CaCl_2$	Substrate
1.19	0.03	0.21	0.74	Tributyrin
0.52	0.06	0.23	0.33	Methyl butyrate

Table III

	0.05N HCl (in cc.)			
Control	0.5% Phenol	0.5% NaF	0.5% Quinine HCl	Substrate
1.06	0.53	0.50	0.19	Tributyrin
0.77	1.64	0.19	0.42	Methyl butyrate

on the esters of the lower fatty acid (butyric) was, however, quite high during early stages of development. A summary of the data concerning the lipids of the grasshopper eggs is of interest. The fat in the egg of *Melanoplus differentialis* is liquid at room temperature (fusion point, 26.2° C.); in *Chortophaga viridifasciata* the fat is solid (fusion point, 39.4° C.) (Slifer, 1930). The former insect spends the winter as an egg, the latter as a nymph. The iodine number of the fats is the same in both animals (135 to 140) (Slifer, 1932). The low melting point in the winter eggs may possibly be due to the higher proportion of short chain fatty acids. This is the explanation of the liquid fat of the aphid, *Pemphigus,* which contains glycerides of butyric, caprylic and lauric acids (Timon-David, 1927–28). The presence of monoesters

rather than glycerol triesters might give similar results. The data concerning the enzymes present in the grasshopper egg lend credence to the assumption that the lower fatty acids are present in the egg. Slifer (1930) has shown that the total fat (measured after saponification by a method for higher fatty acids) decreases only slightly during prediapause (9.7 per cent), yet the volume of the lipoidal layer as measured by Bodine et al. (1939) decreases 32.5 per cent in the same period. Slifer (1930) found a loss of 50 per cent in postdiapause, the volume determinations, 42.5 per cent. The amount of fatty acids in a diapause egg is approximately 8 per cent of the wet weight of the egg (Slifer, 1930) as compared to an amount of lipid equal to 3½ per cent of the wet weight of the egg obtained by the centrifuge separation. The fat obtained by this latter method is a mixture of esters (probably glycerol) which contains C_{12} to C_{18} fatty acids (Allen, T. H., personal communication). Experiments to determine the hydrolysis of the lipid separated by centrifuging and also lipids extracted from the egg brei with petrol ether showed demonstrable amounts of hydrolysis after a 24-hour period only in the case of the latter. This might well be due to the existence of esters and acids in equilibrium.

The relative amounts of hydrolysis in these two enzymatic reactions cannot be quantitatively compared with the activity of esterases from other sources. In general it seems evident that the enzymes are relatively concentrated in the grasshopper egg, since experiments described with other esterases involve periods of four hours and upwards at 37° C. to produce enough acid to be measured. Fiessinger and Gajdos (1936) found that the tributyrinase from the larva of *Galleria mellonella* was much more active than that from human blood serum (ca. 10 times). They also could demonstrate no reaction with olive oil as a substrate.

The two lipolytic enzymes possess strikingly different physical and chemical properties as evidenced by the independent change in potency during development, the inactivation by heat, the effect of temperature on the rate of hydrolysis, the possibility of separating the two enzymes, and the difference in effect of the inhibitors used. Curiously, the tributyrinase, *per se,* is less sensitive to heat treatment than methyl butyrinase yet more susceptible to temperature in the presence of its substrate. This may be due to a reversal of the heat inactivation in the former case.

The evidence indicates the lipolytic enzymes in the grasshopper are present in greatest quantities at the time the egg is laid. From these high levels at the time of least differentiation in the egg the enzymes decrease in amount during development or differentiation (Fig. 1). A change in the amounts of esterase in the egg of the trout (*Salmo fario*) was observed by Falk and co-workers in a careful and detailed study of

this material. Methyl butyrate was not hydrolyzed by the esterase from the unfertilized egg, but the hydrolysis was accomplished by eggs 35 or more days after fertilization. Methyl and ethyl acetates were easily hydrolyzed by the egg but steadily less so as development proceeded; ethyl butyrate showed a reverse effect. The value of esterase action generally was high in immature eggs, small in mature eggs, increasing with development (see Needham, 1931, for summary). In the work of Falk et al. cited here no data are given for the esters of the long chain fatty acids. In the grasshopper egg the decline in the activity of the monobutyrinase after the tenth day of prediapause development occurs somewhat later than the decline in potency of the natural activator (presumably a lipid) of protyrosinase (Bodine et al., 1939). It is possible that some of the substances serving as activators are monoesters of fatty acids and that these are utilized rapidly in early development. Subsequent to this period the amount of monobutyrinase falls. However, the explanation of this effect suggested by Bodine and Carlson (1940) seems more tenable. The decline in the amounts of both enzymes studied during post-diapause development seems correlated with the rapid disappearance of yolk. The possibility that these enzymes may be found in the serosa has not been excluded in these experiments, yet the major part seems to be contained in the yolk and probably is incorporated into the midgut after its absorption. This conforms to the evidence of Stuart (1935) that the yolk cells become part of the midgut just previous to hatching. The cells of the intestinal tract then "inherit" these enzymes from the yolk. Other hydrolytic enzymes may come to be in the gut of the adult in a similar manner.

Summary

1. Glycerol extracts of the grasshopper egg (*Melanoplus differentialis*) have been tested for hydrolytic activity on methyl butyrate, tributyrin and olive oil during various stages in the development of the egg. The ability to hydrolyze methyl butyrate is high when the egg is laid; this value declines between the tenth and fifteenth day of development, remains constant during diapause and slowly declines again during the post-diapause period. The action of extracts on tributyrin is much stronger, remains constant from the time of laying until the cessation of the diapause and then declines markedly. No action on olive oil could be demonstrated.

2. Optimum activity in hydrolysis of tributyrin is at pH 6; the activity of the enzyme reacting with methyl butyrate is only slightly affected by changes in the H-ion concentration.

3. Temperature affected the methyl butyrinase and tributyrinase activity in a different manner. Exposure to temperatures above 55° C. depressed the activity of the former while the activity of the latter persisted to 65° C.

4. The hydrolytic action on tributyrin increased with temperature between 5° and 25° C. and declined at higher temperatures. Methyl butyrinase activity increased with temperature between 0° and 45° C.

5. The esterases seemed to be associated with the yolk until just before hatching.

6. The effect of sodium oleate, calcium ion and various inhibitors of lipolytic enzymes on the extracts used were determined.

The author wishes to express his appreciation to Professor J. H. Bodine for his helpful advice and criticism.

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ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIX. Protyrosinase and Morphological Integrity of Grasshopper Eggs[1]

JOSEPH HALL BODINE AND THOMAS HUNTER ALLEN

(From the Zoölogical Laboratory, State University of Iowa)

Although protyrosinase has been found in extracts of grasshopper eggs, no evidence for its existence within the intact egg has been presented. In view of the possibility that the very process of extraction might inactivate the enzyme, it seems desirable to examine the relation of protyrosinase to morphological integrity. It should be possible to perform such a test by subjecting eggs to one of those treatments which cause the activation of extracted protyrosinase. An increased rate of oxygen uptake and the appearance of melanin in the intact egg should then indicate that protyrosinase had been present before its transition into tyrosinase. This paper deals with results of experiments showing the occurrence of protyrosinase within the intact egg of a grasshopper, *Melanoplus differentialis* (Thomas).

The data which are graphically illustrated in the accompanying figure were obtained from recordings of a Warburg apparatus operated at 24.9° C. The time course of oxygen uptake was plotted for groups of 100 intact eggs which had just previously been heated for five minutes in water kept at certain indicated temperatures. The rates of oxygen uptake of diapause eggs heated between 62° to 85° C. remained constant through the first 100 cu.mm. but declined as a limiting volume of 225 to 230 cu.mm. was approached. However, the rates of oxygen uptake of eggs which had been exposed to temperatures below 50° C. were constant. Relative values for the velocity of oxygen uptake may thus be given by the reciprocal of the time in minutes for the utilization of the initial 100 cu.mm. of oxygen. When these values are compared, a complex temperature effect is found (see figure). It is proposed to interpret this effect according to the properties and occurrence of protyrosinase.

If an egg extract containing protyrosinase is heated for five minutes at temperatures between 60° and 85° C., a tyrosinase is formed (Bodine

[1] Aided by a grant from the Rockefeller Foundation for work in cellular physiology.

and Allen, 1938). Heating seems to affect the stability of both protyrosinase and tyrosinase. With ascending temperature the former is activated, while the latter is destroyed. Consequently, the tyrosinase activity of an extract increases from 60° to 75° but declines from 75°

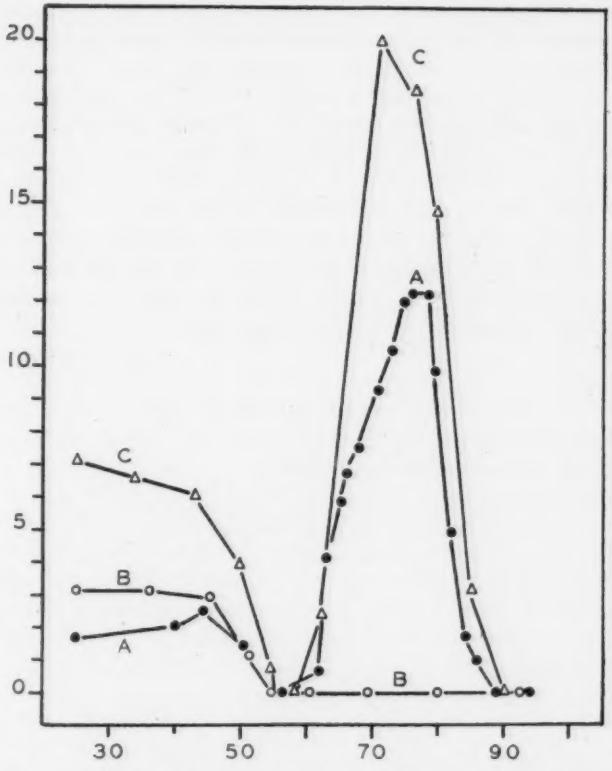

FIG. 1. The effect of heat treatment on the oxygen uptake of grasshopper eggs in various stages of development. Ordinate, reciprocal of the time in minutes for the uptake of 100 cu.mm. of oxygen multiplied by 1000; abscissa, temperatures in °C. to which eggs were exposed for five minutes. Curve *B*, 7-day eggs (prediapause); curve *A*, 60-day eggs (diapause); curve *C*, eggs 3 days post-diapause.

to 90°. A similar differential effect of heat is found for the velocity of oxygen uptake of intact diapause or post diapause eggs (see figure, curve *A* and *C*). Since protyrosinase and a naturally occurring substrate can be extracted from eggs of these stages (Bodine, Allen, and

Boell, 1937), it appears that the increased velocity of oxygen uptake of the intact egg must be due to the heat-induced enzymic oxidation of the native substrate. Curve *C* is presumably higher than curve *A*, because in post diapause there is more native substrate than in diapause (Bodine, Allen, and Boell, 1937).

The latter interpretation also seems to be supported by the eventual formation of melanin, by the low value for the "respiratory quotient," and by the sensitivity to cyanide. Diapause eggs, which six hours previously had been heated between 62° to 84° C., changed from a pale lemon yellow to a dark olive-green color. Upon dissection it seemed that the darker color was due to the presence of a brown pigment—melanin—located in the "liquid-filled space" (Slifer, 1937) between the serosa and cuticle. Similar eggs heated below 62° and above 84° C. remained a lemon yellow, because their protyrosinase supposedly had either not been activated or else had been destroyed. From measurements of the oxygen uptake and carbon dioxide production performed according to the indirect method of Warburg (Dixon, 1934), an R.Q. of 0.1 to 0.2 was found for eggs that had been heated at 75°. Such a value is to be expected during the production of melanin (Raper, 1928). Potassium cyanide in a concentration of 0.01 M abolished the oxygen uptake produced by heat activation. These properties are usually considered to be characteristic of a tyrosinase reaction.

Since protyrosinase has not been found in extracts of eggs younger than eight to nine days of age (Bodine, Allen, and Boell, 1937), one should not expect an increased velocity of oxygen uptake for seven-day eggs that have been exposed to those various degrees of heat sufficient for activating protyrosinase. The occurrence of such a phenomenon would serve essentially as a control experiment for the heat treatment of those eggs containing protyrosinase (see figure, curve *B*). The respiratory processes of prediapause and diapause eggs are evidently susceptible to the effects of heat. Perhaps the normally working respiratory enzymes are destroyed at 56° C. (see figure). If such be the case, it may then be supposed that the portion of the curve for diapause or post-diapause eggs between 62° and 85° C. pertains entirely to the activation of protyrosinase and the destruction of tyrosinase.

The addition of an activator followed by the formation of an enzyme presumably should indicate through cause and effect relations that a proenzyme had once been present. It therefore seems that heat treatment has demonstrated the occurrence of protyrosinase as a constituent of diapause and post-diapause grasshopper eggs. This demonstration of protyrosinase seems to be independent of the trituration and dilution

inherent to an extraction process. Thus it appears that protyrosinase exists within the intact grasshopper egg and that this protyrosinase does not lose characteristic properties as a result of extraction. Moreover, these deductions on the occurrence of the inactive rather than the active enzyme would lead to the conclusion that oxidations coupled with a tyrosinase reaction (Allen and Bodine, 1940) can hardly be expected to complement the respiratory processes of these eggs. Although extracted protyrosinase can be activated by an oil native to these eggs (Bodine, Allen, and Boell, 1937), this lipide is probably bound to various proteins or isolated in such a way that it is inaccessible to the protyrosinase of intact eggs (Bodine and Carlson, 1940).

Summary

Protyrosinase occurs in the intact egg of the grasshopper, *Melanoplus differentialis*, and shows properties similar to those for extracts prepared by trituration of the eggs. Moreover, it seems that protyrosinase, as a naturally occurring entity, is not an artefact produced by extraction procedures.

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THE FOUNDING OF ANT COLONIES

LAURENCE J. LAFLEUR

The normal method of founding colonies among the more typical of formicine species is generally understood to be as follows. The males and females swarm on a given day and copulate in the air, the males subsequently dying. Each female then descends to earth, tears off her wings, and finds a suitable spot for her colony. According to Wheeler [1]: "In her cloistered seclusion the queen now passes days, weeks, or even months, waiting for the eggs to mature in her ovaries. When these eggs have reached their full volume at the expense of her fat-body and degenerating wing-muscles, they are laid, after having been fertilized with a few of the many thousand spermatozoa stored up in her spermatheca during the nuptial flight. The queen nurses them in a little packet till they hatch as minute larvae. These she feeds with a salivary secretion derived by metabolism from the same source as the eggs, namely, from her fat-body and wing-muscles. The larvae grow slowly, pupate prematurely and hatch as unusually small but otherwise normal workers. In some species it takes fully ten months to bring such a brood of minim workers to maturity, and during all this time the queen takes no nourishment, but merely draws on her reserve tissues. As soon as the workers mature, they break through the soil and thereby make an entrance to the nest and establish a communication with the outside world. They enlarge the original chamber and continue the excavation in the form of galleries. They go forth in search of food and share it with their exhausted mother, who now exhibits a further and final change in her behavior. She becomes so exceedingly timid and sensitive to the light that she hastens to conceal herself on the slightest disturbance to the nest. She soon becomes utterly indifferent to her progeny, leaving them entirely to the care of the workers, while she limits her activities to laying eggs and imbibing liquid food from the tongues of her attendants." To this general picture I wish to suggest three types of modification; as to fasting, hazards to the colony, and coöperative founding.

Experiments have shown that queens can live for ten months or more without food, and bring up young in the meanwhile. Experiments have even been made with the precaution of furnishing nothing but distilled water. But it does not follow that because ants are able to fast

[1] William Morton Wheeler, *Ants*, Columbia University Press, 1910, p. 185.

for long periods that they regularly do so, any more than the fact that these same ants can withstand immersion implies that they regularly stay under water for any considerable duration: certainly no one supposes that in nature the queens abide by a diet of distilled water. And there are a number of reasons to believe that queens regularly leave their incipient nests to look for food.

For many years I have kept nests of ants, principally small ones developed from queens taken during swarms or from incipient nests less than a year old. In searching for incipient nests it has been my experience that I find as many queens wandering at large as I find queens in their nests. This does not mean, clearly, that half the queens of incipient nests are away from them at any given time, since the nests may be very hard to find. One day this June, for instance, I spent a few hours in a wooded area looking for young nests under loose bark. Appropriate trees were rare, and most of them were preëmpted by well-established colonies, so that I failed to find a single incipient nest. But there may have been several so well hidden that I did not find them, and there were undoubtedly hundreds of such nests in the soil, where I was not concerned to look for them. If any of these queens left her nest, however, the chances were good that I would spot her, and I did in fact so find a queen of an earth-nesting species that had probably swarmed the previous fall. This and many other similar experiences serve to convince me that it is by no means rare for a queen to leave her nest.

What is the purpose of these excursions? For several days after a swarm queens may be observed in decreasing numbers. Some of these may be late swarmers, but not many, as winged females are not found in comparable numbers. Here the reason is undoubtedly the search for more suitable quarters. During the remainder of the year, queens are less commonly observed, and the motive of their wandering is hunger. Invariably, when these queens are put in artificial nests, their first act is to eat heartily. The queen referred to in the previous paragraph spent more than an hour and a half continuously imbibing honey.

It may well be that many queens, attempting to found a nest on the starvation basis heretofore described, fail, and after consuming all their progeny wander forth to be detected by the myrmecologist. This is directly suggested by the fact that in several recorded experiments where queens failed to rear colonies, they eventually sought to escape. There is also the possibility, more significant if true, that queens may leave their incipient colonies in a perfectly healthy state while they forage for food. In artificial nests they frequently leave their brood to obtain honey or other foodstuffs some distance away within the nest. It should

also be mentioned that a few queens in artificial nests show no interest in food made available to them. In only one case in my experience, however, has the queen died, and as this occurred with a species with which I have been uniformly unsuccessful, the single case of the queen that died without eating is hardly significant in view of the two score that ate and died too. The situation here is artificial, in that no barriers are placed between the queen and the food, and she does not have to tunnel out or tear down a wall as would usually be the case in nature.

One observation of mine, however, throws direct light on this situation. In September, 1940, while waiting for the ferry at Hadlyme, Connecticut, I removed the sole piece of bark from an old fallen log and discovered underneath a typical incipient nest of *Camponotus pennsylvanicus* containing three pupae besides some eggs and small larvae. The queen was absent, but I soon saw her hurrying in a straight line for her nest. She was about two yards away when I first noticed her. Here we have a case where the queen, without the intervention of any artificial circumstances, was absent from her nest, possibly in the search for food, while that nest was in a perfectly healthy condition. On the other hand, a few queens in my own nests have failed to eat food provided within the nest, but these queens have been unsuccessful in every case.

Likewise, one occasionally finds incipient nests with such a considerable quantity of young that it is extremely unlikely that the queens have existed on a starvation basis. For example, in a very populous stump in Arlington, Vermont, I found a number of isolated queens of *Camponotus noveboracensis* that had undoubtedly swarmed the same year. In a few cases I was able to examine the cavity carefully and to take a census that was accurate as to pupae and larvae, although possibly incomplete in the count of eggs. One queen had four pupae, two larvae, and three eggs; another three pupae, three larvae of pupal size, and five eggs; a third three pupae, two large larvae, and fifteen eggs. Pupae and larvae about to pupate have consumed all the food that they eat before they emerge as adults; consequently these queens had provided the food for four, six, and five workers respectively, and had considerable numbers of other young as well. The queen of *Camponotus pennsylvanicus* previously mentioned as having been taken while returning from a foraging trip had three pupae besides a considerable number of small larvae and eggs.

If starvation is less of a hazard to ant queens than has been generally supposed, there is nevertheless a terrific mortality from other causes even when the queen survives the day of the swarm. Many queens must be killed while foraging, or have their nests invaded by other ants

or hostile insects; some succumb to parasites or to fungoid growths; and many must find themselves in such an unfavorable environment that their callow offspring, on emerging, find insufficient food or are killed or captured by other ants.

Besides these obvious perils, experience with a large number of incipient colonies in my artificial nests has indicated the importance of a number of other factors, an importance which seems to me not inferior to that of obtaining food. These factors are the following:

1. There is a very considerable mortality rate among queens during the first few weeks after swarming. This I estimate at 20 per cent, a figure which has held true from species to species, year in and year out, despite the best of laboratory care and even when the queens are adopted by workers of their own species or are given callow young immediately.

2. Some queens fail to take necessary sanitary precautions, fouling their nests and allowing mold to destroy their young and themselves. This is one of the many faults I have noticed in *Prenolepsis* queens.

3. Some queens fail to lay eggs at all; or, having laid some, cease to do so. Unless ovipositing recommences within a month or two this has always led, in my experience, to the death of the queens. Six queens of *Formica subsericea* were taken by me on August 8, 1940. One of them died in the first few weeks, leaving five, of which one failed to lay eggs. She died on January 16, 1941. Another queen of this group experienced difficulty in laying an egg; and on February 5 I observed her bent double for over ten minutes, an egg occasionally visible in her cloacal orifice. Later, however, she became normal in her egg-laying. A third queen of this group was badly mauled by the others on January 2, but was successful with her colony until she ceased laying eggs about February 20. She died on April 18. Of seven *Camponotus novebora-censis* queens taken on July 6, 1941, and previously mentioned in this article, one died on July 11 for no apparent reason, and a second, failing to lay any eggs after being taken, died on July 24.

4. Sometimes queens neglect to collect their eggs, allowing them to die and become moldy wherever dropped. *Prenolepsis* is particularly prone to do this. A similar fault is to drop eggs into crevices whence the queen is later unable to extract them. *Prenolepsis* is again a frequent offender in this way, and I have observed it not infrequently with *Cremastogaster*. Of course, any ant may occasionally lose an egg in a crack, if one exists in the vicinity. A curious incident occurred, however, with one queen of *Formica subsericea*, who developed a mania for hiding her eggs. A number were shoved as far as possible under the rim of a bowl, where she could not extricate them, and others were

hidden individually in wet cotton, where they were forgotten. When moved to another nest and given a worker, this queen became perfectly normal and successful.

5. Some queens eat all their progeny regardless of the presence of other food. In mid-August, 1923, a period when other queens had many larvae, pupae, and even callow young, I took a queen of *Camponotus pennsylvanicus* without young. On July 26, 1941, I took a *Camponotus noveboracensis* queen with only one medium-sized larva. On the twenty-seventh the larva had disappeared and an egg was present, although there was honey and meat in her cell. By July 31 the egg, together with a second laid subsequently, had disappeared; and for the next two weeks eggs were eaten shortly after being laid. A queen of *Formica subsericea* laid four eggs on November 26, 1940. One was eaten that evening. Another was laid on the twenty-seventh, and one eaten November 28. Two more were laid November 29, but all were eaten by December 12. While this article was being written, two affiliated queens of this species devoured the fifteen eggs they had collected, and have not acquired any more in the two weeks since that event.

6. That a few eggs should be eaten to further the development of the more advanced young is natural enough, but I once saw a *Camponotus pennsylvanicus* eat her most advanced larva, and on several occasions larvae have been consumed in the presence of both pupae and eggs; and once or twice pupae have been eaten in the presence of older pupae, as well as larvae and eggs. A *Camponotus ferrugineus* queen and one worker devoured three well-formed naked pupae, but saved a medium-sized larva.

7. In one nest larvae twice failed to grow, either because of some constitutional defect or because they were not fed. This instance is that of the *Camponotus pennsylvanicus* queen taken in September, 1940 and already mentioned in another connection. Of the three pupae, two developed normally and one was stillborn: the small larvae remained small, however, from September until the following June, when all but one disappeared during some affiliation experiments. In this period eggs developed normally into larvae, but failed to grow in the larval stage. The larvae appeared healthy enough, except that their skins were less shiny than normal. At the conclusion of the affiliation experiment this nest contained only the queen and one larva of the queen's first year progeny, two alien workers, and five alien males. A new lot of eggs began to be laid on June 1, just before the experiment, and these started hatching July 17. Once more, however, despite the presence of a new lot of workers, the larvae failed to grow.

8. When larvae pupate and commence to spin, the queens sometimes allow eggs and small larvae to become entangled in the spun silk of the pupating larva, usually with fatal results. This failure might be much more rare under natural conditions, where the pupating larva can be buried in earth. The same *Camponotus* queen previously mentioned invariably put her whole stock of eggs and small larvae on any one of the introduced male larvae which happened to be pupating at the moment. If these were rescued, she restored the status quo ante as soon as the human intervention was over. In fact, it is to this behavior that I ascribe the eventual death of the first-year eggs and larvae some nine months from their first appearance.

9. A not infrequent fault in incipient nests is the failure to open cocoons, though this is the fault of workers more commonly than of queens. One *Formica nitidiventris* queen lost ten out of fifteen cocoons in this way. Four were saved only because I opened them, and the fifteenth was tenderly guarded by the queen, long after its demise was evident (on close application) even to human nostrils. These four were removed to another nest, after which the sixteenth and seventeenth cocoons were successfully opened.

10. It is less easy to understand why cocoons are sometimes partially opened, and the unborn ant allowed vainly to struggle with one leg or antenna protruding, or perhaps a whole head. Yet this happens not infrequently, while the queen and sister workers pay no attention. The third worker of the same *Camponotus pennsylvanicus* queen struggled for forty hours with only its head emerged, before I took pity on it and released it.

11. When the cocoons are opened, the membranes may not be completely removed. As these dry, they contract and twist the still supple exoskeleton into awkward and useless shapes. At best this results in a curving and weakening of a leg—most commonly one of the hind ones—at worst it results in the complete incapacitation of one or more members. Some examples may be given: a *Camponotus noveboracensis* queen left her third worker hampered by membranes around both hind legs, and I removed these membranes thirty-six hours after birth. A *Formica subsericea* colony worked on the tenth callow to be born for six hours without removing all the membranes. A *Cremastogaster* queen left her first worker swathed in membranes for twenty-four hours, after which my efforts to save it were unsuccessful. In a colony that contained three queens of *Formica subsericea,* fighting among the queens prevented proper attention to the young, and several died; one had badly deformed antennae; and two were saved only by my intervention. In the nest of *Formica nitidiventris,* already mentioned, the third worker

born (exclusive of the fifteen cocoons of early vintage) was left with membranes binding one hind leg to the gaster.

12. The infant mortality rate, among ants as among other animals, is higher than the rate at any other period. In a healthy and well-developed nest it may be negligible, with incipient nests I expect to lose between 5 and 10 per cent of callows through stillbirth and death in the first two weeks of life. This estimate does not include losses through failure to open cocoons or remove membranes.

13. The young workers, when they arrive, may be deficient in very much the same ways as the queens. One additional defect, however, is failure to forage. During the nine months that her two workers lived with the *Camponotus pennsylvanicus* queen before mentioned, they failed to seek food, but obtained it instead by regurgitation from the queen.

14. Workers may fail to keep the nest clean. In my experience this is particularly true of *Formica neocinerea,* and of slave-making ants where this species is used as slave. The related *Formica subsericea* is also addicted to this carelessness, although to a lesser extent.

15. Misdirected activity with regard to eggs is not unknown among workers. In a nest of *Lasius* which I collected in 1936, all the pupae were allowed to soak in the water compartment. As this would kill the pupae in short order, I turned out the whole nest. A few hours later one worker began putting the pupae in the water once more, but I removed them and the act was not repeated.

16. Workers may eat the young. In a nest of *Formica subsericea* containing one queen and one worker, the latter ate the eggs laid by the queen despite the presence of other food. She never molested one egg, but would eat any excess over this number. Finally, after the death of the queen, she was left alone with this one, and nursed it to medium-large size. Then she affiliated with another queen, and did not return to her infantivorous practices. In another nest of this species the two workers consumed the only young then present, which consisted of five eggs.

17. In small nests, inexperienced workers are more prone than queens to leave cocoons unopened. In larger nests, one at least of the workers will usually be successful in caring for the young, and the others take their cue from her. In nests started with nothing more than larvae and pupae, some of the latter being opened by hand, it is usually necessary to continue opening operations for some time before the workers take over. Somewhat the same conditions prevail in an incipient nest, and if the queen immediately leaves everything to the first few workers born, which she frequently does, one or two callows are apt to die or be born crippled before the workers become adept at their jobs. In one nest of

Formica subsericea the eighteenth worker to be born was left helpless in a half-opened cocoon and was relieved of her cocoon and membranes by me some ten hours after her birth.

It is interesting to notice, in passing, that when callows are being relieved of membranes, they almost invariably submit willingly to the necessary treatment. When picked up, the callow tries to escape, although its efforts are much less violent than those of a full-grown ant. But as soon as the point of a teasing needle is applied in an effort to remove the membrane, the callow remains quiet without being held until the operation is over. I have known adult ants to act in the same way when I have attempted to remove wax which adhered to them and interfered with their movements.

Another point of interest is that when a queen shows a failure in one aspect of nest-building, that queen and her progeny are very apt to show degeneracy in other ways as well. Consider the case of the *Camponotus pennsylvanicus* queen. Her first worker was the only one born successfully. The second was stillborn; the third required my aid at birth. Though they were ready enough to defend the nest, these two workers failed to aid the queen in caring for the young, or in foraging for food. And in two successive seasons, the larvae failed to grow beyond minimum size. Such a series of mishaps could not occur in nature, for any one might well be fatal, and any two would almost certainly be so.

We now pass to the third and last modification of the usual picture of colony foundation. While queens are capable of founding nests unaided, they may receive aid in doing so, and this method seems to me to be more important than has been generally realized. It is well known that certain species are temporarily or permanently parasitic; and that in other species minims accompany the queen on the marriage flight. It is also known that queens sometimes collaborate in the founding of nests and that a large colony may retain many of its own females as additional queens, but these facts have not been given their full weight. My own experience is that in the two highest subfamilies—and these include all the well-known ants of the north temperate zone—polygynous colonies are just as typical as monogynous. In some cases, indeed, the number of queens is fantastic. Windsor[2] reports that he removed forty-nine dealeated queens of *Formica neocinerea* from one spadeful of earth, and in opening a nest of *Formica sanguinea subintegra* his impression was that the queens were almost as numerous as the workers.

Furthermore, on the day of swarming and for a few days thereafter, the queens of most species are exceptionally ready to form alliances, and

[2] Reported to me by letter and to be published in "Anti-Social Behavior among Ants," *Journal of Comparative Psychology, circa* April, 1942.

any number can be put together successfully. This even applies to queens of different species, if they happen to swarm within a day or so of each other. This was forcefully demonstrated while this paper was being written, for *Lasius* and *Acanthomyops*[3] swarmed on the same day and two boys who had been asked to collect queens for me put sixty of the former and eighteen of the latter in one jar. The group was entirely peaceable, and no casualties whatever resulted from the strange mixture. The rule is not true of all species, however, nor of all queens of any species. When *Cremastogaster* swarmed this year, I collected three groups of six queens each, and one of four. In one group one queen killed all the others; in a second only one queen died, while no injuries occurred in the other two.

An instance is on record where a nest divided into two sections, which gradually separated and became distinct colonies. This process of colony formation is probably quite important: that it is so is indicated by the fact that many nests in a given area fraternize (sic) with each other.

It must happen not infrequently that colonies lose their queens and descend towards extinction. Under such circumstances the workers are exceptionally ready to adopt queens of their own or even of a related species. The same is probably true of colonies whose queens have become infertile, and possibly for colonies that have undergone other discouraging misadventures. When large colonies are deprived of their queen, their morale may be shattered and groups of workers migrate into suitable holes and shelters in the vicinity. In such cases it is possible for several groups to adopt queens and for one colony to aid in the development of several new nests. The evidence for this consists partly of the fact, easily determined by experiment, that queens and workers affiliate more readily than do queens alone or workers alone. It is also easy to show that small, queenless, or demoralized groups of workers affiliate more readily than do normal colonies, and this principle has been successfully applied by me in artificially inducing affiliation. On several occasions, indeed, such groups have actively sought affiliation, as when a group of about two hundred queenless workers of *Tetramorium caespitum* forced their way into an alien colony of fifty workers and several females and affiliated with them. Third, I have noticed in both natural and artificial nests that when deprived of a queen and of young the workers exhibit a tendency to wander, and to congregate in small groups of from five to fifty. And lastly, the well-known behavior of many of the permanent parasites demonstrates that queens can get themselves accepted by another colony.

[3] *Acanthomyops murphyi*, determination by William S. Creighton. The *Lasius* were principally *L. americanus*, with two *mixtus* and one *nearcticus*.

We now come to the last method by which queens may receive aid in founding a colony. In nature, worker ants must occasionally wander so far that they are unable to find their way home, and others must be carried away by wind or water, or be transferred to new areas by clinging to birds, animals, or to human transport. Solitary workers will die in a day or so at most, but I seriously doubt whether the majority of strays die in this manner. It appears to me, on the contrary, that most of these strays will enter other formicaries and there be killed or adopted, largely dependent upon the size of the community entered. If an object being explored by an ant be removed to some considerable distance from her nest, the typical behavior may be readily observed. At first the worker shows the normal exploratory behavior: she examines crevices for food, and will capture any available. If she meets an alien ant she avoids her, but without any appearance of panic. Sooner or later she apparently becomes aware that she is lost: her movements are now more rapid, she usually ignores food (except for an unusually luscious tidbit such as a drop of honey), and she exhibits fright in contact with alien ants. If she is put into another nest during this period she tries to escape but rarely fights back if attacked. Still later another change may be noted: the ant apparently abandons hope of finding her own nest, and there are two conditions frequently met. In one the worker becomes quiescent, with only a quivering of an antenna or leg to indicate life, and dies within a day—sometimes within an hour. In the second typical condition the worker becomes interested in other ants and will actively seek alliances with them. In either case the worker forms alliances readily, although only passively in the first type. Even after making alliances, however, workers of the first type occasionally die or wander off in a few days. It is quite clear that these stray workers, who cannot be excessively rare, form an accessible auxiliary to queens who are founding nests.

I have observed a related type of behavior in—so far—only a single species. Almost any worker of *Camponotus noveboracensis* will affiliate with any solitary queen if she wanders or is put into the latter's nest. On several occasions I have seen such workers feed the queen and care for the young for a day or so, and then seek to leave. This raises the question whether this temporary affiliation can occur at all frequently in nature. Somewhat similar behavior has been previously reported of *Acanthomyops,* which are said to feed alien workers that enter the nest. I have been unable to verify this behavior. If either type occurs, it would almost amount to a confederation of a whole species for mutual aid, a condition previously unknown in the insect world, and infrequent elsewhere.

STUDIES ON EXPERIMENTAL HAPLOIDY IN SALAMANDER LARVAE

II. Cytological Studies on Androgenetic Eggs of Triturus viridescens

CORNELIUS T. KAYLOR

(From the Department of Anatomy, Medical College, Syracuse University and the Marine Biological Laboratory, Woods Hole, Massachusetts)

Introduction

One of the outstanding features of the experiments on androgenesis with eggs of salamanders has been the high rate of mortality during cleavage and gastrulation (Fankhauser, 1934*a*; Kaylor, 1937). However, there have been surprisingly few investigations on the cytology of failure of development in these early stages of androgenetic development. The most extensive observations have been those of Fankhauser (1934, *a* and *b*) on androgenetic egg fragments of *Triton palmatus* and more recently of Fankhauser and Moore (1941) on androgenetic eggs of *Triturus viridescens*. There have been a number of cytological studies on parthenogenesis in eggs of frogs (review of literature, Parmenter, 1933), but these have been concerned more with the rôle of the nucleus in early development of the egg (Dalcq, 1932) or with the origin of diploid and higher numbers of chromosomes (Parmenter, 1933, 1940) in cells of eggs and embryos rather than some of the factors underlying a failure of development beyond certain stages.

In view of the scarcity of studies on the cytology of early stages of androgenetic development in salamanders, the present study seemed to be indicated. It is a survey of the microscopical evidences of the causes of cessation of development in androgenetic eggs of *Triturus viridescens*. A preliminary cytological examination (Kaylor, 1939) showed that an irregular distribution of chromosomes had taken place in these eggs, as in the merogonic eggs of Fankhauser, and was probably responsible for the arrested development, since in this type of experiment no injury to the existing organization of the egg is possible.

Material and Methods

Material

During the course of experiments on androgenesis in *Triturus viridescens* (Kaylor, 1937, and later experiments not published) consider-

able material was preserved for future cytological studies. Of this material, 63 eggs which had ceased development during early cleavage, blastula or gastrula stages were selected for cytological examination. Fifty-nine of these eggs had actually completed their developmental possibilities; they were fixed either after they had remained in the same stage for 12 hours or more or at the onset of cytolysis as indicated by a beginning discoloration of some of the cells. Four of the eggs were preserved because of broken yolk membranes.

Methods

Experimental.—The technique used in obtaining these androgenetic eggs has already been described in detail (Kaylor, 1937). It consists essentially of the removal of the second maturation spindle from the egg by puncturing the polar area containing the spindle with a fine glass needle and sucking a small amount of material into a capillary pipette. The egg then develops with only the male, haploid set of chromosomes.

Fixation, Sectioning, Staining.—All eggs were fixed in Bouin's fluid, cleared from 95 per cent alcohol through wintergreen oil, and imbedded in paraffin containing about 5 per cent bayberry wax. This fixative hardens the yolk, but satisfactory sections were obtained by soaking the imbedded eggs in water for 12 to 24 hours, after the first 10 or 12 sections were cut and mounted: the method used by Fankhauser and Moore (1941). After this soaking, a complete ribbon of perfect sections was obtained. The sections were cut at 15 μ, parallel to the animal-vegetal axis. The sections were stained in Harris' acid-haemalum for the nuclear stain, eosin as a counterstain for the yolk granules, and Light green for the spindle fibers. They were then cleared from 95 per cent alcohol through pure aniline oil and mounted in an aniline-balsam mixture. The use of aniline was necessary since the use of xylene after the staining and dehydration processes always cracked the sections.

Figures 1 and 9 were drawn at a magnification of 80 and reduced to one-half in reproduction.

Observations

Observations on the Living Eggs

To review briefly the former observations on the living androgenetic eggs of *Triturus viridescens* (Kaylor, 1937), it was found first of all that although the majority of the androgenetic eggs underwent irregular cleavage and died prior to gastrulation, this abnormal cleavage was not

entirely responsible for the early cessation of development, since approximately one-half of the normally segmenting eggs failed to develop beyond the gastrula stage. Secondly, there existed no correlation between the type of cleavage of an androgenetic egg and the number of spermatozoa present in the egg at the time of operation. It was obvious, then, that a detailed cytological study of the early development of androgenetic eggs might determine the causes of the early arrested development.

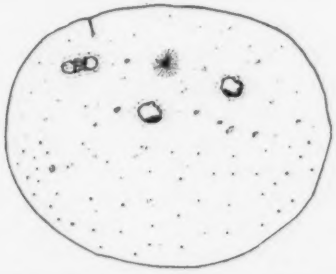


FIG. 1. Drawing of a median section of the egg 30.4e, sectioned parallel to the egg axis. All nuclei projected into this section from neighboring sections. Three degenerating sperm nuclei: two in prophase, one in telophase. One cytaster. Indication of a furrow.

Cytological Observations

The following stages of development of androgenetic eggs were examined in sections:

Stage of Development	Number of Eggs Examined
a. Irregular beginning cleavage	
1. Abortive cleavage	7
2. Early irregular cleavage	5
b. Early blastula	19
c. Late blastula	23
d. Gastrula	9
	Total 63

a. Irregular Beginning Cleavage Stages.—1. *Abortive cleavage.* Seven eggs were fixed 25 to 36 hours after operation, during which time only a few irregular, incomplete furrows had appeared on the egg surface. These furrows were still visible at the time the eggs were preserved. Surprisingly enough, in the sections there was no evidence of furrows in six of the seven eggs (Table I). One egg showed definite irregular furrowing, not connected with mitotic activity within the egg.

In each of these eggs there was evidence of early mitotic activity on the part of the sperm nuclei. The evidence is summarized in Table I. The cytological condition of each egg showed very little variation. The majority of sperm nuclei degenerated either before or after early mitotic activity. Cytasters were present in most of the eggs. Figure 1 illustrates the typical cytological condition encountered. In this particular egg, three or four sperm entrance marks were present on the egg surface at the time of operation. Three degenerating nuclei and

TABLE I

Summary of cytological conditions in abortive cleavage stages

Egg No.	No. Sperm Marks	Age	External Appearance	Cytological Condition
		hours		
26.1e	1	32	Irregular furrows at animal pole	No furrows, 2 nuclei degenerating in prophase, 1 spindle, no chromosomes
27.5e	3	36	Irregular furrows at animal pole	No furrows, 1 nucleus degenerating in prophase
28.6e	3	26	Irregular furrows at animal pole	No furrows, 3 nuclei degenerating in prophase
30.4e	3–4	26	One irregular furrow	Indication of furrows, 2 nuclei degenerating in prophase, 1 nucleus degenerating in telophase, 1 cytaster
30.5e	2	36	Irregular furrows at animal pole	No furrows, 1 cytaster, cytolysis
30.6e	4	25	Irregular furrows at animal pole	No furrows, 1 degenerating nucleus, 4 cytasters
101.1e	6	28	Irregular furrows at animal pole	No furrows, many degenerating nuclei

one cytaster were actually found in the egg; two of the nuclei were degenerating at prophase, and one at telophase.

2. *Early irregular cleavage.* Five eggs, fixed 24 to 26 hours after operation, were examined in this group. The cytological condition of each of these eggs is summarized in Table II.

From this table it is clear that the sperm nuclei in these eggs began to divide at the same or nearly the same time. One nucleus divided sooner than the others, but succeeded in forming only a few small, irregular cells. The "accessory" sperm nuclei either degenerated dur-

ing early mitosis, or continued to divide haphazardly. In any case, the presence of so many constellations in the egg does not lead to the formation of complete cleavage furrows.

Figures 2 and 3 illustrate the cytological condition of two of the most interesting eggs of this group. In the egg shown in Fig. 2, five

TABLE II

Summary of cytological conditions in early irregular cleavage stages

Egg No.	No. Sperm Marks	Age	External Appearance	Cytological Condition
		hours		
32.1e	6	26	Irregular cleavage	4–6 irregular "cells," no nuclei. In unsegmented region: 6 nuclei degenerating in prophase (monaster), 1 cytaster
34.3e	7+	26	Irregular cleavage	6–8 irregular cells, degenerating nuclei. In unsegmented region: 3 nuclei degenerating in prophase (monaster), 3 nuclei degenerating in metaphase (bipolar), 1 degenerating nucleus
61.1e	7	25	Irregular cleavage	6–8 irregular cells, degenerating nuclei. In unsegmented region: 3 nuclei degenerating in prophase (monastral), 1 nucleus degenerating in bipolar mitosis, 2 cytasters
63.1e	6	24	Irregular cleavage	4 irregular cells, mitosis in each. In unsegmented region: 5 nuclei degenerating in prophase (monastral), 9 cytasters, 1 triaster
64.2e	3	26	Irregular cleavage	many irregular cells, nuclei in majority degenerating. In unsegmented region: 4 small bipolar spindles, 7 degenerated nuclei, 3 large triastral mitoses with large number of chromosomes, 2 large tetrastral mitoses with large number of chromosomes, 10 cytasters

of the six spermatozoa entering the egg are degenerating after a beginning monastral mitosis. It is probable that the sixth sperm nucleus divided in a normal manner and was responsible for the formation of the few cells in the upper part of the egg. In four of these cells, a normal haploid mitosis is in progress. The nine cytasters scattered through the unsegmented part of the egg apparently have no connection with any of the sperm nuclei and for this reason probably originated

de novo in the cytoplasm, as they do in egg fragments of *Triton* (Fankhauser, 1934*a*). The large triaster is probably a fusion of three cytasters.

The cytological condition of the egg in Fig. 3 is much more complex. An inventory of the contents of this egg is given in Table II and in the explanation of Fig. 3. Since there were only three sperm entrance

FIG. 2. Drawing of a median section of the egg 63.1e, sectioned parallel to the egg axis. All nuclei and cytasters projected into this section from neighboring sections. Irregular cells, mitosis in each. Five degenerating sperm nuclei, nine cytasters, one triaster in the unsegmented yolk region.

FIG. 3. Drawing of a median section of the egg 64.2e, sectioned parallel to the egg axis. All nuclei projected into this section from neighboring sections. Irregular cells, degenerating nuclei in most cells. In the unsegmented region: four small bipolar mitoses, seven degenerated nuclei, three large triastral mitoses and two large tetrastral mitoses with large numbers of chromosomes, ten cytasters.

marks on the living egg, it seems probable that only two sperm nuclei could have been responsible for the large number of irregular mitotic figures present, while the third sperm nucleus initiated the formation of the few small, irregular cells in the upper part of the egg. Several chromosome counts were made in the figures present in the yolk region.

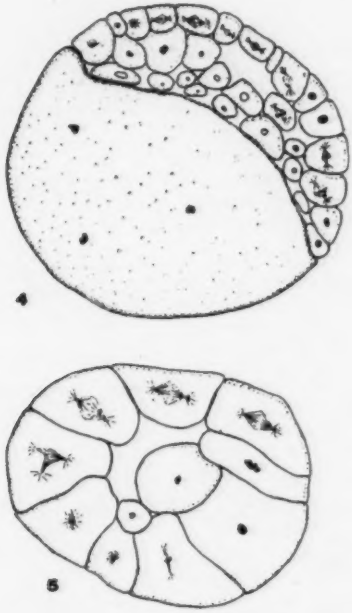


FIG. 4. Drawing of a median section of the egg 28.5e, sectioned parallel to the egg axis. Nuclei projected from neighboring sections into the cells and unsegmented region. Many cells non-nucleated, some with single asters, others with degenerating sperm nuclei in the yolk region.

FIG. 5. Drawing of a median section of the egg 30.7e, sectioned parallel to the egg axis. Nuclei projected from neighboring sections into the cells. Majority of cells non-nucleated. Fairly normal blastula.

In one normal anaphase figure, seventeen chromosomes were identified; eight at one pole and nine at the other (Fig. 10). In another anaphase, 18 chromosomes were identified, while in a nearby metaphase plate, 13 chromosomes could be counted. Several large, irregular triasters and tetrasters were in this yolk region. Large numbers of chromosomes were present in each of these figures.

b. Early Blastulae.—Nineteen eggs were fixed approximately 28 hours after operation, when they failed to develop beyond the mid-blastula stage. The most conspicuous features of the sections of these eggs were, first, that in 13 eggs a large area of the vegetative region was unsegmented. Only 6 eggs were completely segmented. Secondly, closer examination revealed a large number of abnormal mitotic figures

TABLE III

Chromosome numbers in early androgenetic blastulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers									
		9	10–11	11	11–12	12–14	14–16	16–18	18+	22	22+
26.9e	6				2		2				2
20.21e*	6				1	2		1			2
28.5e	2	1				1					
34.2e	25		2	3	5	6	2	2	4	1	
37Ae	11				2	1		1	1		6
52Ae	6				1	3		2			
53Ae*	6				1	1	2	2			
54.3e	12		1	3	8						
68Ae	2					2					
70.2e	11	3	3	3	2						
208.16e	8					4		2			2
00.E	18				8	3		3			4

* Preserved while still developing.

in the cells and unsegmented regions; in the 19 eggs a total of 312 mitoses of the following types were observed:

(*a*) Pluripolar mitoses.
(*b*) Monastral mitoses.
(*c*) Bipolar mitoses with degenerating chromosomes.
(*d*) Bipolar mitoses with no chromosomes.

Figure 4 is an example of an egg of the group of thirteen eggs with the undivided vegetal region. The cytology of this egg is given in detail since, although the egg does not possess all of the irregularities listed above, it is in general illustrative of the cytological condition of this group of eggs. A large sector of the roof of this blastula is composed of cells without nuclei; each cell contains a small bipolar spindle with no chromosomes. Other cells nearby contain only a single aster. In a few cells, bipolar mitoses are in progress, but in several of these figures chromosomes are showing signs of degeneration. Figure 11 il-

lustrates an anaphase spindle in one of these cells. Four chromosomes are lagging on the spindle and show definite abnormal swelling. The large unsegmented yolk region of this egg contains three nuclei which are degenerating in early prophase. The undivided yolk region of similar eggs, however, contained a larger number of abnormal figures

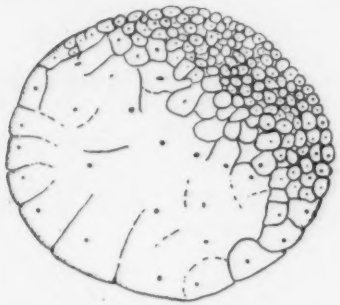


FIG. 6. Drawing of a median section of the egg 25.3e, sectioned parallel to the egg axis. Abnormal late blastula. Large areas of the yolk region unsegmented. No blastocoele.

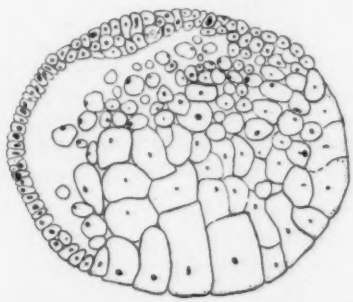


FIG. 7. Drawing of a median section of the egg 23.2e, sectioned parallel to the egg axis. Abnormal late blastula. Cells with pycnotic nuclei in segmentation cavity.

than are seen in this egg. Figures 12 and 13 show two of these mitoses.

The egg shown in Fig. 5 illustrates the typical condition in the group of six completely segmented eggs. The majority of cells contain bipolar spindles with no chromosomes; spindles similar to the one shown in Fig. 14. A few cells contain single cytasters. In other cells, the nuclei are degenerating. The small blastocoele has a few fragments

of cytoplasm containing no chromatin. The other five eggs did not have as many cells without nuclei.

Chromosome counts were possible in some of the cells of twelve of the nineteen eggs (Table III). In all but one of the eggs, the counts deviated from the haploid number (11 chromosomes) in the majority of cells. The one blastula which was haploid happened to have been fixed while still developing. It is doubtful that this egg could have reached an advanced stage of development because the cleavage was very irregular.

TABLE IV

Chromosome numbers in late androgenetic blastulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers									
		7–8	9–10	10–11	11	11–12	12–14	15–18	22	27+	30–33
23.2e	23	12	2				3	6			
25.3e	17	1		1	1	2	3	8	1		
25.9e	22	2	2	4	2	3	5	4			
26.5e	40	5	1	4	14	10		3	1	2	
26.6e*	40			14	15	8				2	1
27.1e*	26			3	5	5	9	4			
36.1e	19	1		4	6	6	1	1			
61.5e	11	1		3	5			2			
64.1e	11	2	7	2							
86.2e	9					1	2	4	1	1	
AA.e	11			1	1		2	7			
3Ae	15	1	1	4	2		2	4	1		
56Ae	13	2	3	3	3			2			
60Ae	12	1	1	4	1	1	2	2			

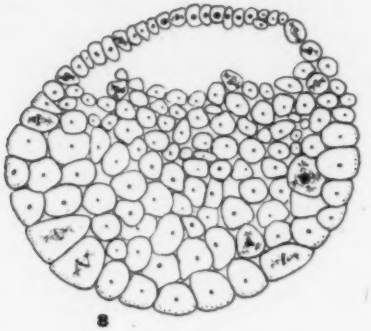
* Preserved while still developing.

c. Late Blastulae.—Of the 23 eggs fixed in the late blastula stage, only two were fixed while still developing. The following description will cover first of all the 21 eggs which had ceased development.

Although in external appearance each of these eggs resembled a normal blastula, the sections showed that all eggs were abnormal. Eighteen eggs were incompletely segmented in certain areas of the vegetal region. Only three eggs were completely segmented. All of the irregularities of mitosis observed in the earlier cleavage stages could be identified in the cells of these blastulae.

Since it would be impossible to describe the cytology of each of these eggs, the blastula shown in Fig. 6 was selected as representative of the group of 18 incompletely divided eggs. No blastocoele is present in the

egg. The upper half of the egg is composed of regularly segmented cells, while in the lower half the boundaries of many of the cells are incomplete. In sixty or more cells, the nuclear conditions were abnormal. The nuclei in the majority of these were degenerating, and in



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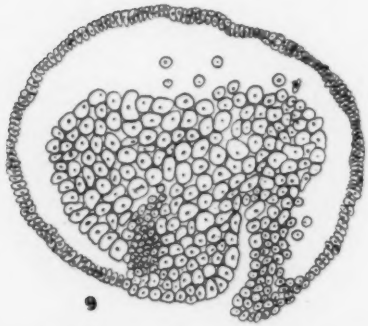

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FIG. 8. Drawing of a median section of the egg 26.6e which was preserved while still developing. Sectioned parallel to the egg axis. Fairly normal late blastula. Irregular mitoses beginning. Tetrastral mitosis in cell of vegetal region at right of drawing, one triastral mitosis in nearby cell.

FIG. 9. Drawing of a median section of the egg 37.3e, sectioned parallel to the egg axis. Abortive gastrula. Incomplete invagination of the yolk. Many cells with pycnotic nuclei in the blastocoele.

other cells, mitoses, still in progress, were frequently of a monastral type. Chromosome counts in 17 cells varied from 7 to 22 in number, indicating that irregular distributions of the chromosomes had occurred earlier in the cleavage history. Several mitotic figures in this egg showed stages of chromosome elimination. Figure 15 illustrates a

metaphase figure in which all of the chromosomes have degenerated. In another cell (Fig. 16), the chromatin is completely removed from the spindle. Other mitoses were observed in which the elimination of chromosomes was occurring more gradually; a few chromosomes at a time were being lost from the spindle. This is seen in Fig. 17. At least two and probably six chromosomes are not included in the metaphase group and will remain outside the nucleus in one of the two daughter cells. A telophase in a cell from another egg (Fig. 18) shows several degenerating chromosomes near the new cell membranes. These chromosomes will not be included in the daughter nuclei.

Each of the three completely segmented eggs possessed a segmentation cavity. Figure 7 illustrates one of these blastulae. About one-

TABLE V

Chromosome numbers in androgenetic gastrulae

Egg No.	Total Number of Analyzable Mitotic Figures	Chromosome Numbers										
		7–8	9–10	10	10–11	11	11–12	12	12–14	15–17	20–21	22+
26.11e	10										7	3
31.1e	14			14								
35.1e	12					8	4					
35.2e	18				6	10	2					
35.9e	6									4	2	
37.2e	25			2	1	7	3	9	1	2		
37.3e	21	1	2	2		7	4	2		3		
75.1e	10		2	2		2		4				
76.3e	9	2	2		1				1	3		

half of the roof of this blastula is composed of a double row of cells. The vegetal region still has abnormally large cells. A number of cells with pycnotic nuclei have separated from the yolk into the blastocoele. Although abnormal mitoses were not observed, an irregular distribution of chromosomes had occurred in earlier stages of development since chromosome numbers in 23 cells varied from 7 to 16 or 18.

Even though development was at a standstill in most of these eggs, mitoses were still frequent. The chromosomes of metaphase plates could be counted accurately in 14 eggs (Table IV). From Table IV it is clear that none of these blastulae were completely haploid.

Two late blastulae were preserved because of ruptured yolk membranes. One of these, Fig. 8, is most interesting because, unlike the majority of operated eggs, its cleavage had been undelayed and perfectly normal. There had been no suspicion, therefore, that the female nucleus

was actually out of the egg. In Fig. 8 it is seen that the egg was a fairly normal blastula. The first few chromosome counts were all haploid. Then the following mitoses were observed: (*a*) two normal bipolar figures with 22 chromosomes (the diploid number); (*b*) one triatral mitosis with 33 chromosomes (Fig. 19); and (*c*) a tetrastral figure with a large number of chromosomes, presumably the tetraploid number. One other cell (Fig. 20) contained a telophase figure with fragments of chromosomes at the center of the spindle. In view of the small number of cells with slightly irregular cytological conditions, this egg could probably have developed to a more advanced stage. The other egg possessed irregular chromosome numbers in the majority of cells. For this reason it probably would not have developed farther.

d. Gastrulae.—The last group of androgenetic eggs consisted of nine eggs fixed at the end of their development in the gastrula stage. In section, all of these eggs were found to be abortive gastrulae. The process of invagination of cells into the blastocoele was incomplete. In most of these eggs yolk cells with pycnotic nuclei were accumulating in the blastocoele (Fig. 9).

Although mitoses were not frequent in these gastrulae, a few chromosome counts were made in each egg (Table V). In all but two eggs, the majority of cells were not haploid. It is interesting to note that one gastrula had only 10 chromosomes in every cell clear enough for analysis. Apparently the lack of even one chromosome may be sufficient to disturb the processes of differentiation occurring for the first time at gastrulation.

The abnormal gastrulation of the two eggs which were completely haploid is not surprising since in later stages of development, as for example the formation of the neural plate, haploid embryos frequently have serious difficulties. This was observed in an earlier report (Kaylor, 1937), and in the experiments on the androgenetic development of frog embryos (Porter, 1939).

Discussion

The cytological conditions found in these eggs explain fully the high mortality rate during cleavage and gastrulation. In eggs fixed after irregular beginning cleavage, it was observed that either none of the sperm nuclei was sufficiently active to form cleavage furrows, or, quite the opposite, all of the sperm nuclei divided at the same or nearly the same time causing incomplete and irregular cleavage of the egg. The cytological conditions were somewhat the same in eggs which ceased de-

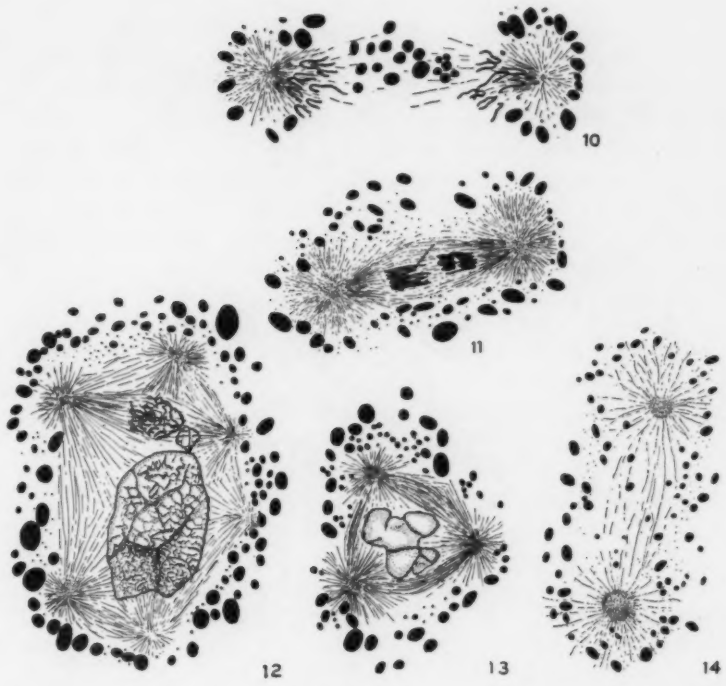

PLATE I

EXPLANATION OF FIGURES

Figures 10 to 14 were drawn at a magnification of 1200 and reduced to ca. 400 in reproduction.

FIG. 10. Anaphase figure in the yolk region of the egg in Text Fig. 3. Nine chromosomes at the upper pole and eight at the lower.

FIG. 11. Anaphase figure in a cell of the egg in Text Fig. 4. Four chromosomes, lagging on the spindle, show beginning degeneration.

FIG. 12. Pluripolar figure in the yolk region of the egg 37Ae. Apparently the fusion of several nuclei.

FIG. 13. Triastral figure in the yolk region of the egg 37Ae. Degenerating nucleus.

FIG. 14. Bipolar figure without chromatin in a cell of the egg 20.21e. The spindle shows a reduction in the number of spindle fibers.

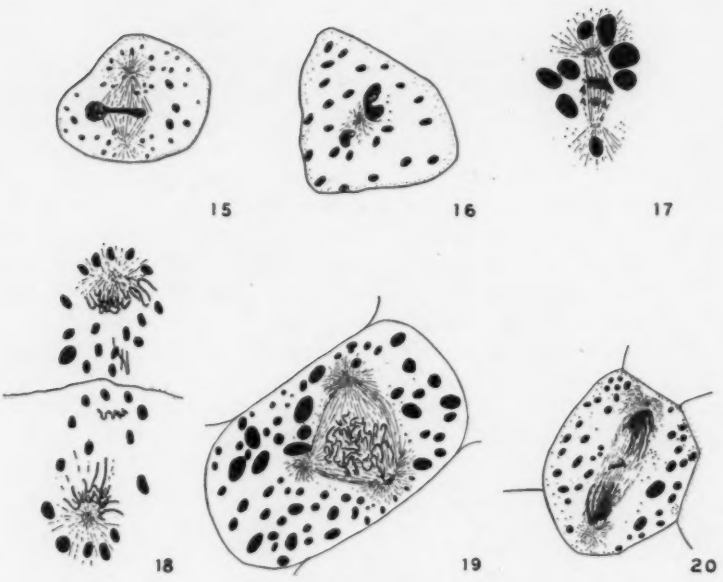

PLATE II

EXPLANATION OF FIGURES

Figures 15 to 20 drawn at a magnification of 1200 and reduced to ca. 600 in reproduction.

Figures 15 to 18, different stages in elimination of chromatin.

FIG. 15. Metaphase figure in a cell of the egg in Text Fig. 6. The chromosomes have degenerated into a pycnotic mass on the center of the spindle.

FIG. 16. Metaphase figure, polar view, in a cell of the egg in Text Fig. 6. The chromatin is completely removed from the spindle.

FIG. 17. Metaphase figure in a cell of the vegetal region of the egg in Text Fig. 6. Six chromosomes lagging on the spindle.

FIG. 18. Telophase mitosis in the egg AA.e. Several chromosomes lagging near the new cell membranes.

FIG. 19. Triastral figure in a cell of the egg in Text Fig. 8. Thirty-three chromosomes present.

FIG. 20. Anaphase figure in a cell of the egg in Text Fig. 8. Several chromosomes lagging on the spindle.

velopment during the early blastula stage. The majority of these eggs were incompletely segmented and contained abnormal nuclei in the cells and in the undivided areas, indicating the early irregular division of more than one sperm nucleus. The few completely segmented blastulae, although fairly normal in their cleavage, were, nevertheless, very irregular in their nuclear conditions. In these cases, the division of only one sperm nucleus probably initiated the almost normal cleavage, but even this early mitosis must have been extremely irregular.

The majority of eggs which had ceased development during the late blastula stage were incompletely segmented in certain areas of the egg. Only a few were normally formed blastulae. Chromosome counts in these eggs showed conclusively that irregular distribution of the male chromosomes had occurred earlier, and, indeed, was still going on in many cells at the time the eggs were preserved. It was of interest to note in the case of the normal androgenetic blastulae preserved while still developing, that one of these eggs possessed irregular chromosome numbers in the majority of mitoses analyzed. In the other egg, it was observed that irregular mitoses were just beginning. Cytological conditions such as these in normally developing androgenetic blastulae would be of importance in experiments involving the transplantation of haploid cells.

The nuclear conditions of the gastrulae were abnormal. About 80 per cent of the eggs ceasing development in this stage were not haploid. All of these gastrulae were abortive. Since it has been shown previously (Kaylor, 1937) that all androgenetic embryos which develop beyond the gastrula are haploid, it is apparent that the early gastrula is as far as an androgenetic egg can develop unless it possesses at least the haploid number of chromosomes in all of its cells.

These observations are in exact agreement with Fankhauser's (1934, *b*) conclusions from his excellent analysis of chromosome numbers and chromosome individuality in andro-merogonic *Triton* eggs. A complete discussion of the indispensability of a balanced set of chromosomes in early development is found in Fankhauser's papers.

These experiments on androgenesis have recently been extended to eggs of the Japanese newt, *Triturus pyrrhogaster* (Kaylor, 1940). In this species, a smaller percentage of the operated eggs die during blastula or gastrula stages. The more normal development of these eggs as compared with that in *Triturus viridescens* must be connected, then, with a more normal behavior of the sperm nuclei in early cleavage.

SUMMARY

1. Androgenetic eggs of *Triturus viridescens* most frequently cease development in the following stages: *a.* Irregular beginning cleavage; *b.* Early blastula; *c.* Late blastula; *d.* Gastrula.

2. The causes of arrested development were investigated cytologically in eggs fixed in each of these stages.

3. Eggs of the first group were of two types, i.e., abortive cleavage, and early irregular cleavage in which a few cells were formed near the animal pole. In seven eggs of the first type, it was found that the sperm nuclei had degenerated either before or during early mitosis and cleavage furrows had disappeared. In five eggs of the second type, either all sperm nuclei had degenerated during early mitosis or one sperm nucleus divided more or less normally while "accessory" sperm nuclei either degenerated or divided irregularly in the unsegmented part of the egg.

4. In nineteen early blastulae, thirteen were incompletely segmented and six, although irregularly segmented, were fairly normal blastulae. Associated with these abnormalities in the thirteen eggs were the independent division of sperm nuclei in the yolk region without segmentation of the cytoplasm, and the presence of abnormal mitoses in the majority of cells. In the six almost normal mid-blastulae, the greater number of cells contained abnormal nuclei. Chromosome counts varied from 9 to 22 + in twelve of the nineteen eggs in which analyses could be made.

5. In twenty-three late blastulae sectioned, the same abnormalities as found in the earlier blastulae were observed. The majority of eggs were incompletely segmented and all of the eggs contained abnormal mitotic figures in some of the cells. Chromosome counts were made in fourteen eggs. None of these blastulae were completely haploid.

6. Nine gastrulae examined were abortive. No abnormal mitotic figures were found in these eggs, but in seven gastrulae the chromosome numbers varied above and below the haploid number, indicating that abnormal mitoses had occurred during earlier cleavage stages. Two gastrulae were haploid and it is assumed that these are examples of the abnormalities which many haploid embryos exhibit when differentiation of parts or of structures first takes place.

7. These observations confirm and extend those of Fankhauser and of Fankhauser and Moore. In order to develop beyond the gastrula stage, an androgenetic egg must be at least completely haploid.

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REVERSAL OF SEX PRODUCTION IN MICROMALTHUS

ALLAN SCOTT

(From Union College, Schenectady, N. Y. and the Marine Biological Laboratory, Woods Hole, Mass.)

Introduction

There is only one known example of paedogenesis in the Coleoptera and there are relatively few cases in the whole insect class. Hence any information which relates to the nature of paedogenesis in the beetle *Micromalthus debilis* has a general biological importance. One important question relative to paedogenesis in the beetle *Micromalthus* is: what is the mechanism which determines the strict separation of male production from female production in two types of larval mothers? This paper shows that the mechanism has an environmental rather than a genetic basis.

Many groups of animals produce unisexual broods. Thus some, aphids, Hymenoptera, Diptera, certain rotifers and Isopoda and some nematodes etc., produce broods of one sex and in some cases a partial explanation of the mechanism of this unisexual propagation is known. In many cases the broods are consistently female and involve a more or less constant process of diploid parthenogenesis, but in a few cases unisexual male progenies also occur. In the genus *Sciara,* Metz (1931) has disclosed a genetic basis which determines the sex of brood and a sex-linked gene is responsible. The thelytokous wasp, *Nemeritis canescens,* studied cytologically by Speicher (1937) is a perfect example of constant female production; no males were found in some fifty generations! The mechanism here also appears to have a genetic basis. It controls sex of progeny by determining a constant type of maturation. In the paedogenetic fly, *Miastor metraloas,* unisexual broods are apparently almost inviolably the rule and although Gabritschevsky (1928) ascribes this to a genetic mechanism, the contrary conclusion is indicated by Ulrich's (1936) work on *Oligarces.* Ulrich has shown that in the paedogenetic fly, *Oligarces paradoxus,* many broods show unisexual propagation although some broods contain both sexes. Hereditary differences among the larvae of *Oligarces* are not the determiners of the sex of the brood. It is the environment that is of primary importance.

It is the physiological state of the presumptive paedogenetic mother and the environment which primarily determine the sex of the brood in *Micromalthus,* just as in *Oligarces.* It is the purpose of this paper to describe a reversal of sex of brood which can be made to occur experimentally in the larval male-producer of *Micromalthus.* All the members of the first brood are male and all of the second brood are female.

Review of the Reproductive Types

In a previous paper (1938) I have described extensively the life history of *Micromalthus* and have outlined the reproductive anatomy of the various types. It is necessary for the purpose of discussion to review the reproductive types and more especially to describe the male producer with considerable care.

There are in the American variety of *Micromalthus debilis* five sexually mature reproductive types: (1) an adult female, (2) a female-producing paedogenetic larva, (3) a male-producing paedogenetic larva, (4) an adult male, and (5) a paedogenetic female larva with a mixed brood. This last is a modified male-producing larva and is the subject of research reported here. It is essential to note that the modified male-producer (amphoterotokous female) is simply a later developmental stage of the male producer. They rarely occur in nature but can be produced in large numbers experimentally. It is of incidental interest to refer here to the apparent absence of male-producers in the South African variety of *Micromalthus* which has recently been reported by Pringle (1938).

Normal History of the Male Producer

The male-producer (arrhenotokous female) is the only source of the adult male. She arises viviparously from a female-producing paedogenetic mother (thelytokous paedogenetic female) as one member of a large brood, sometimes twenty or more. In the first instar all these viviparous larvae possess legs which are lost at an early moult. They are all identical in appearance, indeed, it is impossible to distinguish the male-producer from other types until shortly before the last moult when inspection by dissection shows an ovary of a very special character in the male-producer. This early ovary is often recognizably distinct when only about 80 microns in length when a few egg cells (from one to five in each ovary) first begin to grow (Speicher, 1937). These continue to grow until they are of relatively large size and have become the shape of a hen's egg. The eggs of the thelytokous paedogenetic female are elongate so that the sex of the embryo resulting from either type

egg is predictable long before maturation. This adds another animal to the list showing sexual dimegaly of the ova (Wilson, 1925). When they are mature, the eggs of the male-producer begin development by haploid parthenogenesis in contrast to the diploid parthenogenetic development of the viviparous young (Scott, 1936). The one male that is successful in emerging from the mother is shed as a very young embryo in late June or early July. It is most peculiar, however, that although several embryos may be present in the ovary, only one is born. This new-born male remains for some four or five days adherent to the outside of the mother as is shown in Fig. 2 of Plate I. By that time he has developed sufficiently to insert his head into her genital aperture, which is shown at the arrow in Plate I, Fig. 1. Within a few days more the male has devoured his mother completely. These canabalistic males pupate and soon emerge as male adults.

This astonishing form of reproduction raises several perplexing questions. (1) Why is but one embryo shed by the male-producer when others equally advanced in development are present? (2) Is any one of the embryos more likely to be born than any other; viz., (*a*) does the position of the male in the mother have any bearing on successful emergence? Or (*b*) does the age of the embryo affect his ability to emerge? (3) Can any one of the other embryos be shed if the one that has been born is not allowed to feed upon the mother? (4) What becomes of the male-producer if her son is prevented from eating her?

The Birth Process

Why is only one male embryo shed by the male-producer? I can give no answer to the question but can only indicate some additional facts. Only seven male-producers in a group of three hundred and fifty-seven have given birth to two embryos. Fifty-eight male-producers have shed their male embryos in isolation. The females had previously been placed each in a shallow depression made in black wax and kept in a moist chamber. It is apparent from this that removal from their gallery in the wood does not affect their ability to give birth to the male embryo. Four of the fifty-eight individuals which shed in isolation,

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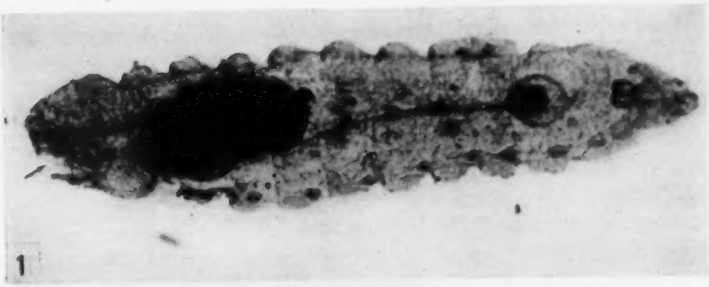
PLATE I

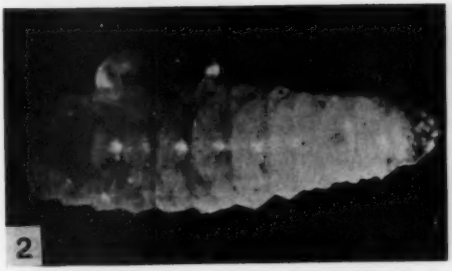
Fig. 1. Feulgen preparation of male producer with one egg visible. The genital aperture is shown at the arrow.

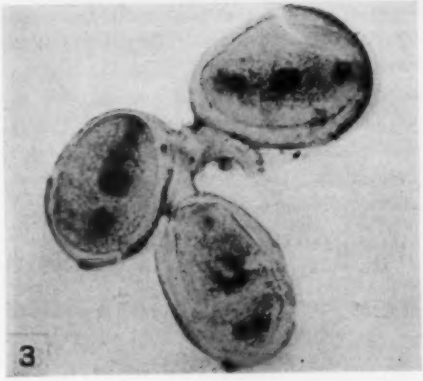
Fig. 2. Male producer and successful male offspring.

Fig. 3. Ovary of the male producer with three embryos, all in the same developmental stage.

Fig. 4. Ovary of the reversing male producer with a newly-developed female embryo, and an exceptionally well-developed male embryo still within its follicle.







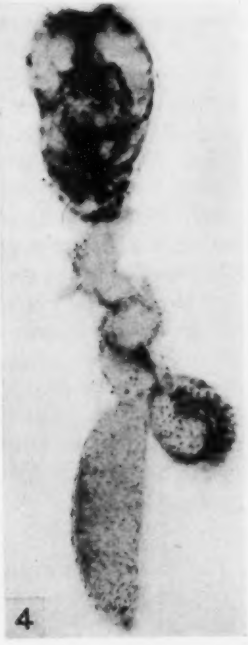


PLATE I

shed two eggs, thus it is possible that removal from the wood favors the birth of a second embryo.

Two other factors might conceivably affect the birth process, that is,—(1) position in the mother and (2) stage of development of embryo within the mother. I have previously shown that there is no favored position in the ovary from which an embryo is shed. (Scott, 1938, Fig. 13.) The successful male embryo may have occupied any position within the ovary. Indeed, the successful embryo may sometimes occupy such a position within the mother that it must experience some mechanical difficulty at birth, since other embryos appear to block its exit.

I do not think that the most mature male embryo is necessarily the most likely to emerge, for frequently the difference in age of the embryos is negligible, as is shown in Plate I, Fig. 3, and moreover, an embryo may occasionally develop into a rather well-developed larva while still within the follicle of the ovary, as illustrated in Plate I, Fig. 4.

No factual explanation of the mechanism governing this uniparity is available. However, a very plausible hypothesis can be formulated from the point of view of natural selection. The male has no other source of food during his larval life than his mother's body and since a second male would compete for this food supply, a process may have been developed which prevents this competition. This process very probably involves the active coöperation of the mother in that some internal physiological mechanism prevents a second male from being born. This mechanism is quite conceivably a failure of the muscular contractions which normally expel the egg. The continued presence of the born male on the mother is not necessary to prevent birth of the remaining embryos. The birth of one in some way sets the mother against further activity of the ovary and ducts. Does a hormone govern the contractions involved in the ovulation-birth process?

A New Brood in Male Producers

The fate of the mother after the emerged male has been removed is quite unexpected and is, I believe, a quite unprecedented observation. In practically every surviving case after approximately four weeks time a new, small brood is born. The members of the new broods have not yet been reared, nor have chromosome counts been possible, but the offspring are judged to be females with considerable certainty for the following reasons: (1) the shape of the egg is in every way similar to that of the female-producing female, (2) the development is in every way identical with that of the ordinarily produced females, and is vastly

different from the development of the male embryo, (3) the appearance of the newly-hatched embryos is identical with that of the more normally produced female larvae. That is, these second brood embryos possess well-developed legs and well-differentiated jaws, whereas new-born male

TABLE I

	Dead Dissected 7/24	Alive Dissected 7/25	Total	Percentage
Females showing female embryos on dissection	1	43	44	21.8
Females showing no female embryos	47	2	49	24.3
Females in which ovaries were not located	13	4	17	8.4
Died before examination			91	45.2
Total number involved in experiment—201. Mortality—75.7%				

embryos are rarely beyond the germ band stage. The second brood larvae will, therefore, subsequently be referred to as females.

It should be stressed that this process is not an occasional one but is quite normal for those larval mothers that survive long enough. Thus in the summer of 1938, 93 females from which the male had been removed gave rise to a new female brood. Of this number, 21 mothers shed their brood and the rest showed female embryos on dissection. Since mortality records were not kept in 1938, the experiment was repeated in 1939.

On July 1, 1939, 201 females, each with the shed male removed, were isolated in black wax depressions in Syracuse dishes, 20 to each

TABLE II

New brood born before July 24	4
Found with female brood on dissection 7/24	10
Dead when dissected, ovaries disintegrated	8
Dead, no new brood developing	2
Lost	1
Total	25

dish and kept in a moist chamber. The mortality was severe, therefore the larvae were dissected before they could have given birth to their new brood. Table I summarizes the results.

In another experiment 25 male-producers were removed from the wood and the adherent male was removed from each one. They were likewise placed in a moist chamber at 35° C. $\pm$ 1°. The results are shown in Table II.

In this group 56 per cent developed a new brood of female embryos.

It is impossible from these data to decide whether or not every male-producer can, under favorable conditions, give rise to a new female brood but it is certainly indicated by the fact that out of the 63 animals that were still alive at the end of their respective experiments, only 6 did not show indications of a new brood. It is reasonable to expect that the larvae that died during the course of the experiment would also have given rise to a female brood had they survived.

The mortality, high in both experiments, is less severe at higher temperatures. The difficulty is largely due to the susceptibility of the larvae to mold. Perhaps a sterile technique would obviate the trouble.

Apparently no structural feature of the male producer prevents viviparity of the new female brood since a considerable number have been kept long enough to allow normal birth. The birth process is in every respect similar to that which takes place in the normal female-producing, paedogenetic female.

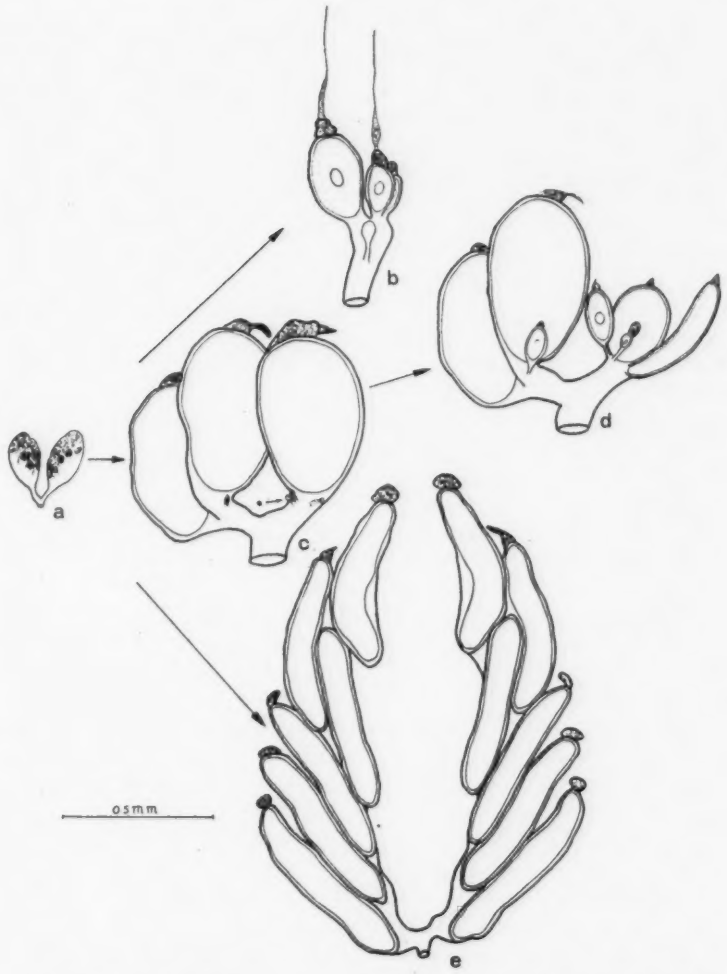
The size of the second brood of the reversing male-producer is intermediate between the size of normal female broods and the size of male broods. Normal female broods are frequently more than ten while male broods are rarely as many as four. An examination of the ovaries of forty reversing male-producers showed that the average number of new eggs formed was 4.2.

Study of the ovaries of this same group of forty reversing male-producers showed that a few females *failed to shed even one male, yet they developed a new brood of female larvae notwithstanding.*

The production of a new brood is not, therefore, absolutely dependent upon the experimental removal of the emerged male from his mother. Male-producers whose emerged male embryo dies will evidently give birth to a new all-female brood in the natural course of events. Indeed, in August, 1937, I found eighteen individuals with a new brood developing, obviously the result of this natural event. Dissection of these eighteen larval mothers showed an empty follicle from which a male had emerged and apparently died.

It seems altogether possible that a third brood might be produced by the original male-producer if it lived long enough. However, a single individual that lived thirty days after the production of the second brood showed no sign of new eggs when dissected.

It will be of some interest to test similarly the further reproductive capacity of the thelytokous paedogenetic female after the birth of her first brood.



TEXT FIG. 1. A diagrammatic representation of the developmental possibilities of the basic ovary as it occurs in the several reproductive forms: (*a*) basic ovary (schematic) showing several undeveloped ovarioles in each ovary; (*b*) the ovary of the adult female with three ovarioles developing; (*c*) the ovary of the male producer (three ovarioles and eggs greatly enlarged, four others have remained small, see asterisk); (*d*) the ovary of the reversing male producer experimentally produced (the rudimentary ovarioles have enlarged and are developing female-producing eggs; (*e*) the ovary of the female producer with numerous ovarioles developing.

Histology of the Reversing Ovary

Studies of the ovaries of the male-producer indicate that the cells which give rise to the new crop of female-producing eggs are already present on the oviducts of the male-producer before the male embryo is born; indeed, they were probably present at the time of the first differentiation of the male-producing ovary. In the mature ovary of the male-producer these cells are located in little clusters around the ventral and lateral surfaces of the oviducts at the point of junction of oviduct and follicle. (Text Fig. 1*c* at the asterisk and Plate II, Fig. 5*b*). Structurally these groups of cells are undeveloped ovarioles. They doubtless represent ovarioles which did not enlarge during the first period of development of the male-producing eggs. The detailed structure of these ovarioles is shown in Plate II, Figs. 5 and 6. In both figures some differentiation can be seen within the ovariole and although no single egg cell can be identified with certainty, still, terminal cells of the germarium, nurse cells, and duct cells can be seen in Plate II, Figs. 6*a*, 6*b*, and 6*c* respectively.

In *Micromalthus* the ovaries of the four reproductive types are fundamentally similar. The general plan of the ovary in each of the female reproductive types is meroistic and acrotrophic, since the nutritive cells are all located at the apex of the ovariole. The variations in structure which the ovaries of the several reproductive types present may all be considered as modifications of a basic, undifferentiated ovary illustrated in Text Fig. 1*a*. This basic ovary possesses multiple ovarioles at the ends of a forked oviduct; it is the development or non-development of these potential ovarioles that determines the nature of the mature ovary. If the ovary develops within an adult female, then three or four of the ovarioles will enlarge with their contained eggs, as indicated in Text Fig. 1*b*. When, however, the basic ovary develops within a female-producing paedogenetic mother, a number of eggs, each in a different

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PLATE II

Fig. 5. Frontal section of ovary of male producer: (*a*) Follicle; (*b*) undeveloped ovarioles (the follicle on the right side appears in another section); (*c*) oviduct; (*d*) last ganglion; (*e*) vagina.

Fig. 6. Oil immersion photograph of undeveloped ovarioles of the male-producing ovary. No enlargement has as yet taken place: (*a*) germarium; (*b*) potential nurse cells; (*c*) potential duct segment; (*d*) oviduct.

Fig. 7. Total Feulgen preparation of a reversing male-producing ovary: (*a*) unshed male egg; (*b*) female-producing egg; (*c*) oviduct; (*d*) new segment of oviduct; (*e*) empty follicle (out of focus); (*f*) vagina or terminal duct.

Fig. 8. Total Feulgen preparation of the ovary of a male producer with a new brood of female embryos: (*a*) female embryo; (*b*) empty follicle; (*c*) retained degenerating male embryo.

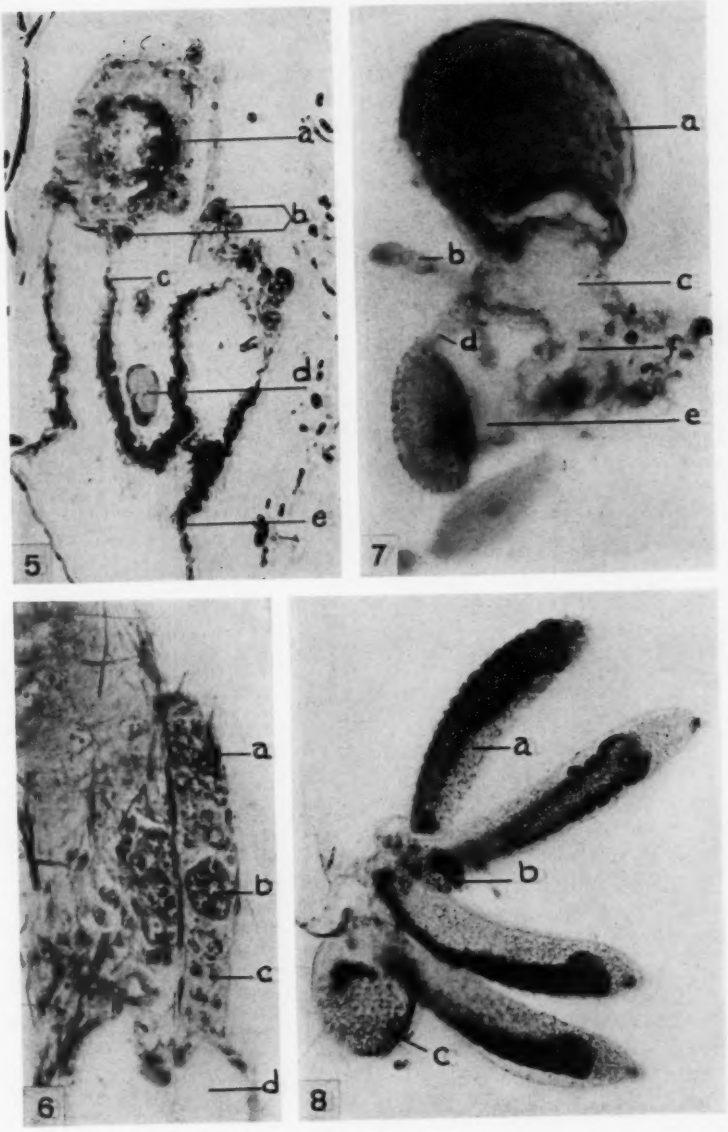

PLATE II

ovariole, enlarge to determine the characteristic ovary of that type of larva. This is illustrated in Text Fig. 1*c*. When the basic ovarioles develop within a male-producer, again only a few of the ovarioles enlarge. In this case, too, only a few eggs develop, one in each ovariole, so that the fully developed ovary of the male-producer, shown in Text Fig. 1*c*, still possesses a number of undeveloped ovarioles at the base of the enlarged follicles. It is these undeveloped ovarioles that enlarge to give rise to the second all-female brood under the conditions noted above and illustrated in Text Fig. 1*d*.

The development of the new eggs involves the production of other new parts of the reproductive system, for although the old oviducts and vagina are utilized by the larvae of the second brood on emergence, it will be apparent from Plate II, Figs. 6*c* and 7*d*, that a new segment of oviduct is added during the development of the new crop of eggs. The potentialities of the ovariole tissue are such, therefore, that it gives rise to the following reproductive structures: (*a*) oviduct, (*b*) follicle cells, (*c*) eggs, one per ovariole, (*d*) nurse cells and (*e*) germarium. The new oviduct segment is at first relatively long, but it is inconspicuous in late development, as Plate II, Fig. 8 shows. Perhaps it is incorporated into the follicle as the egg grows.

It should be added that the development of the new eggs is not particularly related to the stage of development of the retained males, for the latter may be in any stage of development from a post-maturational stage to a well-developed larva. Frequently, indeed, the retained males undergo an abnormal type of development which also has no apparent effect on the new brood.

Summary

1. The paedogenetic, arrhenotkous female in the beetle, *Micromalthus debilis* (Leconte), gives birth to but one male embryo, although unshed males also develop.

2. Factors which determine this uniparity are still uncertain, but neither greater age nor more favorable position in the mother are determining factors.

3. When the single successful male offspring is not allowed to devour his mother, a new crop of eggs develops in the ovary.

4. These new eggs are all of the elongate female type. They develop into a larva identical in appearance with the first stage larva of the thelytokous paedogenetic female.

5. Histologically the new eggs originate from undeveloped ovarioles which failed to develop during the first period of growth of the male-producing eggs.

6. Sex of brood in *Micromalthus* is obviously determined by environment, intrinsic or extrinsic, and not by the hereditary constitution of the mother.

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THE TIME-TEMPERATURE RELATION OF DIFFERENT STAGES OF DEVELOPMENT

FRANCIS JOSEPH RYAN

(*From the Department of Zoölogy, Columbia University, New York City*)

The phenomenon of development appears as a series of processes which are visibly unlike. Many investigators have demonstrated that some of these events can also be separated on the grounds that their rates possess different temperature coefficients. For example, the rate of growth of the gill filaments in the frog is more depressed by a low temperature than is the rate of body growth (Atlas, 1935; Doms, 1915). Again, the rate of embryo formation in *Salmo* has a higher temperature coefficient than the rate of growth in wet weight (Gray, 1928). In view of this and other evidence it has always seemed curious that development could yield approximately the same differentiated product over a wide range of temperatures. As a possible solution to the problem, Tyler (1936*a*) has shown that in some marine invertebrates the temperature coefficients of various cleavages are not only the same but are also identical with those for later stages of differentiation. Yet his results are not comparable with those on the frog obtained by Hertwig (1898) and Krogh (1914) whose data show, although the authors do not point it out, that the temperature relation of cleavage is different from that of later development. The experiments on the egg of the frog to be reported here were designed to discover the temperature relations of some of the more clear-cut events of development which could be accurately measured.

Rana pipiens from Vermont were caused to ovulate at 15° by pituitary injection. Batches of about 25 eggs were shed into finger bowls and fertilized artificially. The sperm suspension was replaced after 5 minutes by 200 cc. of 10 per cent Ringer's solution at the temperature at which the eggs were to be kept and the bowls were then distributed to constant temperature environments. When the jelly swelled, the egg mass in each container was cut into bunches containing about 5 eggs apiece. The 10 per cent Ringer's was replaced daily by solution at the same temperature. Cold rooms, water baths and incubators were used to maintain constant temperatures. Generally the temperatures were constant within 0.1° C. (except at 10° and 8.5° where ice-boxes were used; the maximum observed variations here were 1.0° and 0.5° re-

spectively). The temperature in the finger bowls seldom changed more than 0.5° during observation on the stage of a binocular microscope and observation never lasted as much as 5 minutes. Mortality during early development was less than 5 per cent and development was normal at temperatures between 25° and 8°. Above and below these temperatures mortality increased and abnormalities became frequent, so that at 29.6° usually less than 50 per cent of the eggs hatched and abnormalities were very common.

The times to various cleavages, gastrulation and gill circulation were measured from fertilization for embryos remaining constantly at one of the several temperatures. Cleavage was considered begun when the first slight furrowing was seen on the surface of the egg; gastrulation when the dark line of pigment associated with the initial dorsal lip invagination appeared; and gill circulation when the initial blood corpuscles could be seen circulating in the anterior gill. When the critical time approached, repeated observations were made until about 50 per cent of a batch of eggs had reached the initiation point, at which time it was considered that the stage was entered. All of the embryos in a group of 25 entered a stage well within 10 per cent of the total time necessary to reach that stage. The maximum deviation of any batch of eggs from the average time was about 10 per cent.

In order to portray the relation between the times to different stages at different temperatures a semi-logarithmic plot was chosen (see figures). The logarithm of time was placed along the ordinates and the abscissae represent either temperature, in which case the curves are for different developmental intervals, or stages, in which case the curves are for different temperatures. The choice of one of these abscissae was made so as to employ the largest number of points per curve. In either event it is possible to compare what types of function of time the stages are at different temperatures. Spacing of temperatures along the abscissa was obtained by plotting the data for one stage as a straight line. This arbitrary abscissa was then used as a base for the times to other stages. Stages were spaced along the abscissa in a similar fashion. This method is preferred to the comparison of temperature coefficients inasmuch as: (1) it does not entail a selection of points but involves all of the data; (2) it avoids attributing one of the several controversial numerical constants to the temperature relation; and (3) the linear arrangement of points obtained by a distortion of one axis permits immediate visual comparison of the time-temperature relation.

Figure 1 compares the temperature relation of the different stages of development in *Rana pipiens*. The curve for time between gastrulation and gill circulation (Stages 10–20) has the greatest slope. The curve

for time between fourth cleavage and gastrulation has a lesser slope which is, however, greater than the slope of the curves for all the cleavages. These differences are real. If the curves actually were parallel to that for gastrulation to gill circulation, a time error of 25 per cent would have to be assumed at both ends of the gastrulation curve, an error of about 45 per cent at both ends of the curve for first cleavage and an error of about 45 per cent at both ends of the curve for second, third and fourth cleavage. Such errors are highly improbable because the

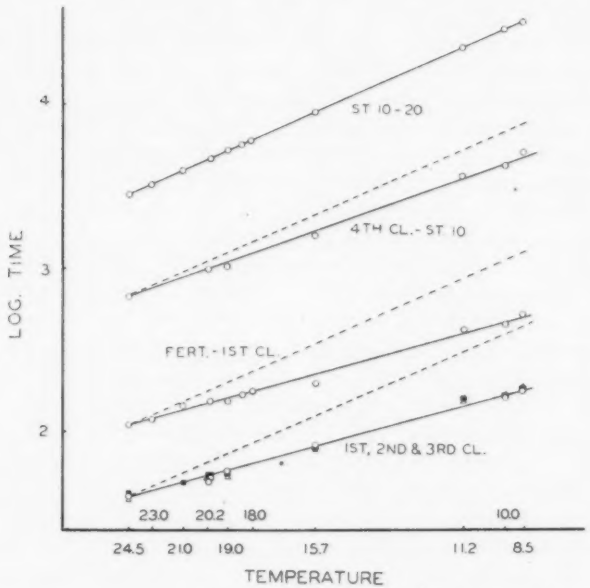

FIG. 1. The relation, at different temperatures, of developmental intervals to time in *Rana pipiens*. Ordinate, logarithm of time in minutes between the specified stages; abscissa, temperature in °C. The data for development between stages 10 and 20 are plotted as a straight line by the arbitrary distortion of the temperature axis. The latter is used as a base for the data for other intervals. The upper complete curve describes development between gastrulation (stage 10 of Pollister and Moore, 1937) and gill circulation (stage 20); the next, between fourth cleavage and gastrulation; the next, between fertilization and first cleavage. In the lowest curve, the circles describe development between first and second cleavage, the squares, between second and third, and the triangles, between third and fourth. Twenty points are single determinations; the remaining thirty-four points are the averages of from two to ten determinations. One hundred and seventy-two determinations were made in all. The broken lines were drawn through the points at 24.5° parallel to the curve for stages 10 to 20. These broken lines emphasize the real nature of the slope differences among the curves for different intervals.

maximum deviation of a point from any one of the curves is only 10 per cent. The deviations of points for the cleavages from the parallel curves drawn through them are at random and are about the size of the expected experimental error (10 per cent). Hence, in *Rana pipiens* the temperature relations of cleavages are alike, but they are different from those of later development.[1] Differences in temperature relation are apparent even in rate-temperature plots (Ryan, 1941) where, in addition to a difference in μ values, the curve for later development shows a "break" at about 18° C., while the curves for cleavage "break" around 14° C.

The data of Krogh (1914) for *Rana butyrhina* when placed on the semi-logarithmic plot (Fig. 2) completely confirm this difference between cleavage and later development. Even though the times to later stages are from fertilization and must include some time during cleavage when the temperature relation is like that of the upper curve, a significant difference in slope is visible. If all the curves actually were parallel, an error of about 25 per cent must be postulated at both ends of the cleavage curve. Such errors are extremely improbable with Krogh's method. His precision in measuring cleavages should be better than that for later stages and yet no such errors are visible in his later stage data (for example, the maximum deviation of a point from the straight lines in Fig. 2 is equivalent to an error in time of only 5 per cent). Despite this difference between cleavage and later development, the parallelism of curves among different stages of later development in Fig. 2 shows that the temperature relations of the latter are alike.[2] Apparently in contradiction to this, Bělehrádek (1926) has calculated for Krogh's data a series of b values increasing from 1.76 to 2.52 between medullary groove closure and 7.8 mm. tadpole formation. However, from the same data and for the same stages b values of 1.6 and 1.7 (and Q_{10}'s decreasing from 4.2 to 3.5) can be calculated according to the points selected for comparison.

[1] Atlas' (1935) Fig. 8 indicates the same temperature characteristic over the low temperature range for the rates of different stages of development in *Rana pipiens*, but the column he uses to include all the points obscures the difference between the temperature relations of the different stages. At higher temperatures the temperature relations of cleavages, of gastrulation, and of later development show the same sort of differences as are visible in Fig. 1 of this paper. Neither the data for *Rana pipiens* described in this paper nor Atlas' data (Fig. 2) for the same animal show the adaptation in the rate of later cleavages found by Hoadley and Brill (1937) in *Arbacia* and *Chaetopterus*, although this may be because the temperatures used were not close enough to the maximum.

[2] Times from fertilization or first cleavage, instead of the length of developmental intervals, are used in this and all subsequent figures in order not to exaggerate errors in timing the events of later development which are difficult to measure.

Confirmation of the similarity in the temperature relations of events in later development can be found in semi-logarithmic plots of the data of Moore (1939) for *Rana pipiens, R. sylvatica, R. clamitans* and *R. palustris,* and of Knight (1938) for *Triton alpestris.* The curves for

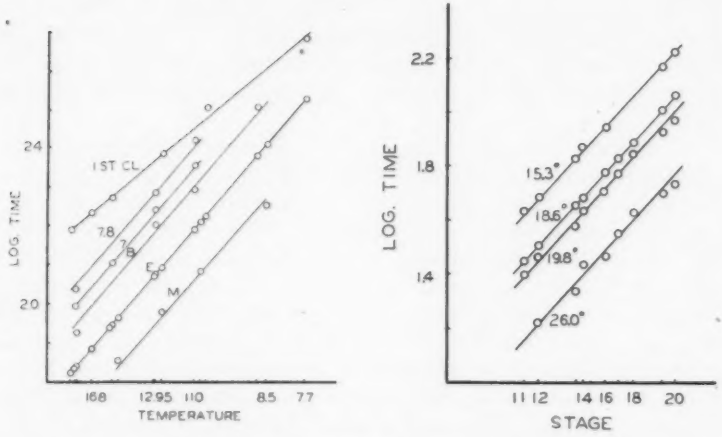

FIG. 2 (left). Krogh's (1914) data for *Rana butyrhina* showing the relation at different temperatures of stage to time from fertilization.[3] Ordinate represents the logarithm of time from fertilization (for cleavages in minutes, for later stages in hours); abscissa, temperature in °C. The symbols over the curves represent first cleavage, 7.8 and 7 mm. length, branched gills, external gills, and medullary groove closure. For the sake of ready comparison, the data for the formation of external gills are plotted as a straight line by the arbitrary distortion of the temperature axis. The latter are used as a base for the data for other stages.

FIG. 3 (right). Moore's (1939) data for *Rana pipiens* showing the relation, at different temperatures, of stages between gastrulation and gill circulation to time from first cleavage. Ordinate represents time from first cleavage in hours; abscissa, stage of development (Pollister and Moore, 1937). For the sake of ready comparison the data for 18.6° are plotted as a straight line by the arbitrary distortion of the stage axis. The latter are used as a base for the data from other temperatures.

later development are all parallel. For example, in Fig. 3 Moore's data for *Rana pipiens* are presented. These supplement the data in

[3] It should be pointed out that the similarity in temperature relation among these stages does not *necessarily* imply that each step in the formation of a given stage has the same temperature relation. There may be differences of short duration which might be in opposite directions and cancel one another. At any rate, if there are such differences, they are not additive, for the overall sort of examination made does not reveal them. The stages in between gastrulation and stage 20 are not clear-cut enough to obtain easily sufficiently accurate determinations to solve this problem.

Fig. 1 inasmuch as they show that many different events between yolk plug and gill circulation have the same temperature relation in *Rana pipiens*. Indeed, the significance of the difference between the temperature relation of the period from fourth cleavage to gastrulation and that of the interval between gastrulation and gill circulation (Fig. 1) is dubious. The process of gastrulation might have the same temperature relation as other stages of later development, but when the measured time also includes cleavages (between fourth cleavage and gastrulation), the observed overall temperature relation would be intermediate. Assuming that the curve for fourth cleavage to gastrulation is just such a composite, a calculation shows that the cleavage temperature relation would prevail into early blastula stages. However, it is very difficult to measure blastula stages accurately enough to break this period into its components and settle the problem.

Peter (1905) computed the Q_{10}'s for Hertwig's (1898) data on *Rana fusca* and claimed that the temperature coefficients gradually increased with the age of the animal. However, when the Q_{10}'s between 15° and 24° are compared for different cleavages, there is no significant difference from the average of 1.37. The same holds for later stages where there is no significant difference from the average of 2.36. Peter's difficulty resides in the fact that he included Q_{10}'s computed for low lethal temperatures where development began but was never completed. In this range the difference between high and low temperatures becomes progressively greater with age. When Hertwig's data are put on a semilogarithmic plot (Fig. 4), it can be seen from the parallelism of curves that the times to stages of later development are the same type of function of temperature but are a different type from that for times to the first three cleavages. Hertwig could have a 25 per cent time error at both ends of each of his three cleavage curves. But since the maximum deviation of a point from the straight lines in Fig. 4*A* is only 10 per cent, the difference between cleavage and later development is probably real. The displacement of the points for gastrulation from the curves in Fig. 4*B* may be real (at 10° there is a time discrepancy of 20 per cent). This is in accordance with the fact shown in Fig. 1 that the rate of gastrulation increases to a degree intermediate between cleavage and later development with a temperature rise. Since gastrulation is compared with cleavage in Fig. 4*A*, the difference between the temperature relations of cleavage and later development is all the more convincing. In summary, it is definite that in Amphibia not only is the temperature relation different for cleavages and later stages, but there are extremely long periods during cleavage and during embryo formation over which the temperature relation remains constant.

It is not surprising that a difference should exist between the temperature relations of cleavages and morphogenesis because visibly these phenomena are unlike and probably have different causes. The amazing thing is the similarity in response of so many different stages to temperature. Second, third, and fourth cleavages are enough alike so that it is not hard to believe that they are the result of the same process. Between fertilization and first cleavage, however, there occur the completion of the second maturation division, release of the second polar

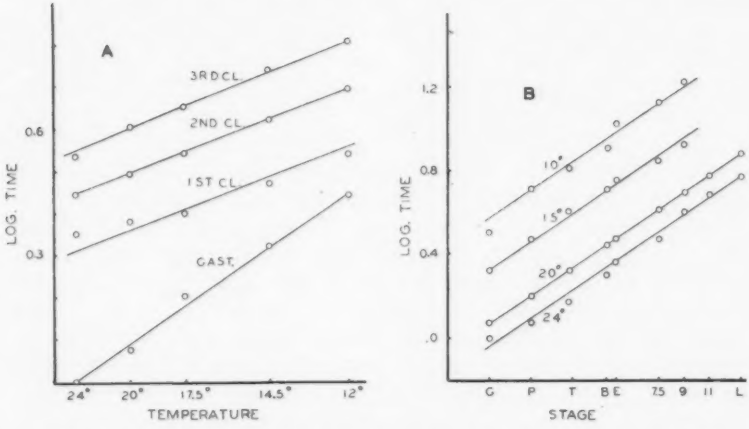

FIG. 4. Hertwig's (1898) data for *Rana fusca* showing the relation, at different temperatures, of stage to time from fertilization. *A*. Ordinate represents logarithm of time from fertilization (for cleavages in hours, for later stages in days); abscissa, temperature in °C. *B*. Ordinate, logarithm of time from fertilization in days; abscissa, stage of development from gastrulation to limb bud formation. At temperatures of 6° and below the parallel relation does not hold, but also development does not go to completion at these temperatures. The abscissae in *A* and *B*, using the data for second cleavage and for 20° respectively as the base curves, have been distorted as in Figs. 1 and 3.

body, and the fusion of pronuclei, before the process begins to resemble later cleavages. Since the total process of first cleavage is, then, different from that of later cleavages, it would be expected *a priori* that the temperature relations should differ. But they do not (Fig. 1). The coincidence may be chance or due to the independence of the cytoplasmic cleavage (which is being measured) from the nuclear phenomena (wherein lies the difference between first and later cleavages) or due to a fundamental process which controls all of the phenomena and imposes its temperature relation upon them. The latter seems more likely

inasmuch as it affords an explanation of the even more astonishing similarity in the temperature relations of stages between gastrulation and stage 20 (Fig. 3). Here such strikingly different processes as neurulation, tail bud and gill formation, onset of circulation etc. have the same relation. Either each operation independently has achieved this or all are controlled by an underlying process which imposes its temperature relation upon its various expressions. Atlas (1938) has shown that in *Rana pipiens* the temperature coefficient of the rate of oxygen consumption during development is approximately the same as that of the rate of development. In many marine eggs temperature affects the rate of early development and the rate of respiration in the same way (Tyler, 1936*b*). These correlations suggest that the "primary gear shaft" (Needham, 1933) integrating developmental processes is some part of the respiratory metabolism.

If this were so, then there should be a difference in the type of metabolism during cleavage in the frog's egg from that prevalent during morphogenesis. Brachet (1934) has, indeed, shown that in the frog the respiratory quotient changes abruptly at gastrulation from about 0.7 to 1.0. Again, in Tyler's (1936*a*) studies of marine invertebrates, there should be the same type of metabolism during cleavage as during later development because the temperature relations of both processes are the same. Accordingly, in *Urechis* where the temperature coefficients of the first four cleavages are the same (Tyler), the respiratory quotient remains unchanged over an equivalent period of time (2½ hours at 20° C.) (Horowitz, 1940). Thus there is real evidence for the belief that the coördinator of the various processes of differentiation, the factor which permits development to be reproducible over a wide range of temperatures, is the respiratory metabolism.

Summary

1. Stages in embryo formation among Amphibia between yolk plug and gill circulation have similar time-temperature relations.

2. The time-temperature relation of cleavage, although constant from first to fourth cleavages, differs from that of embryo formation.

3. It is suggested that the different time-temperature relations of cleavage and of morphogenesis represent different controlling processes; while the similarity of the time-temperature relations among cleavages and among stages of later development is the expression of a common controlling process in each case. These controlling processes are probably parts of the respiratory metabolism and they prevent temperature from disorganizing development.

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